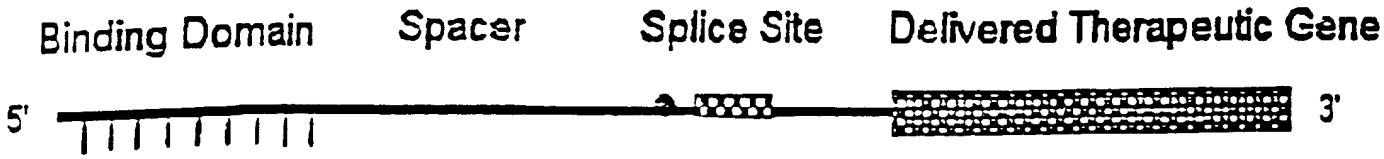
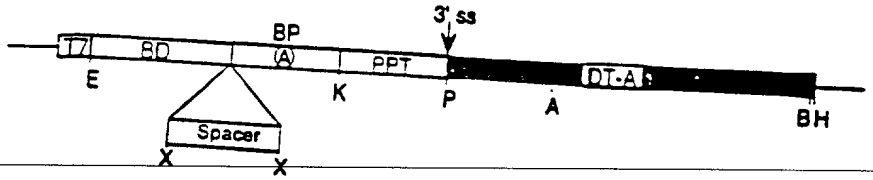
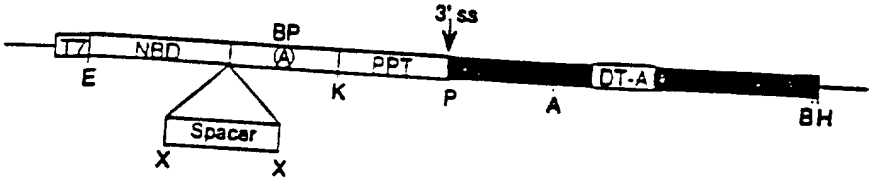
FIGURE 1A



(B) (1) pPTM+Sp



(2) pPTM-Sp



(C)

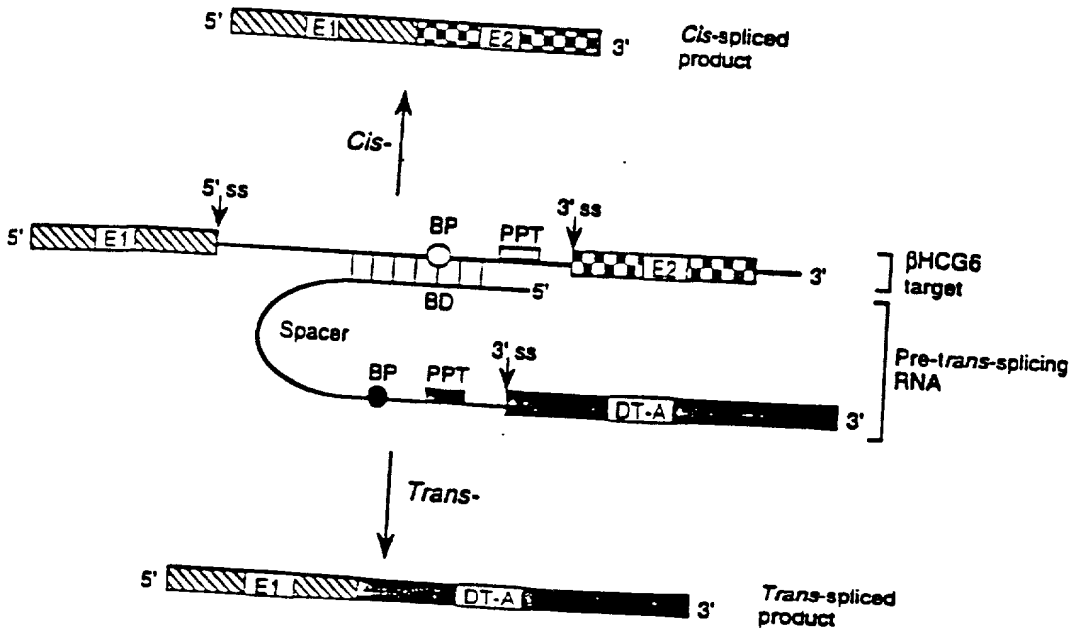
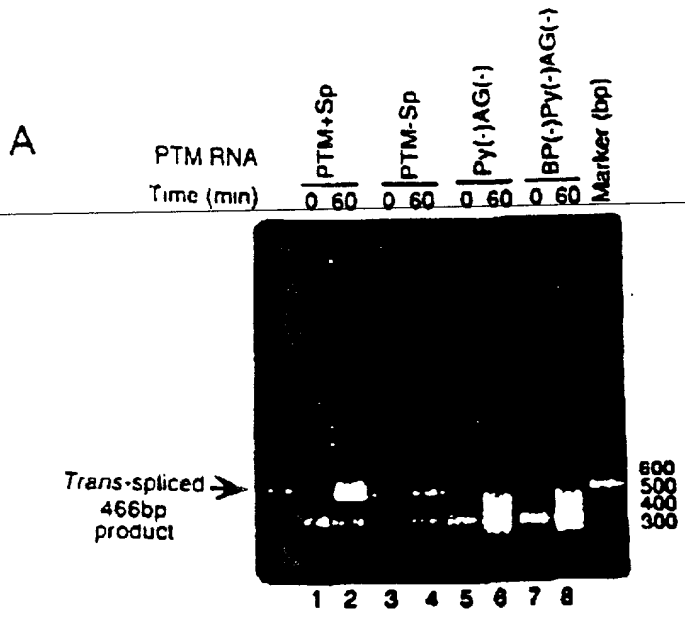
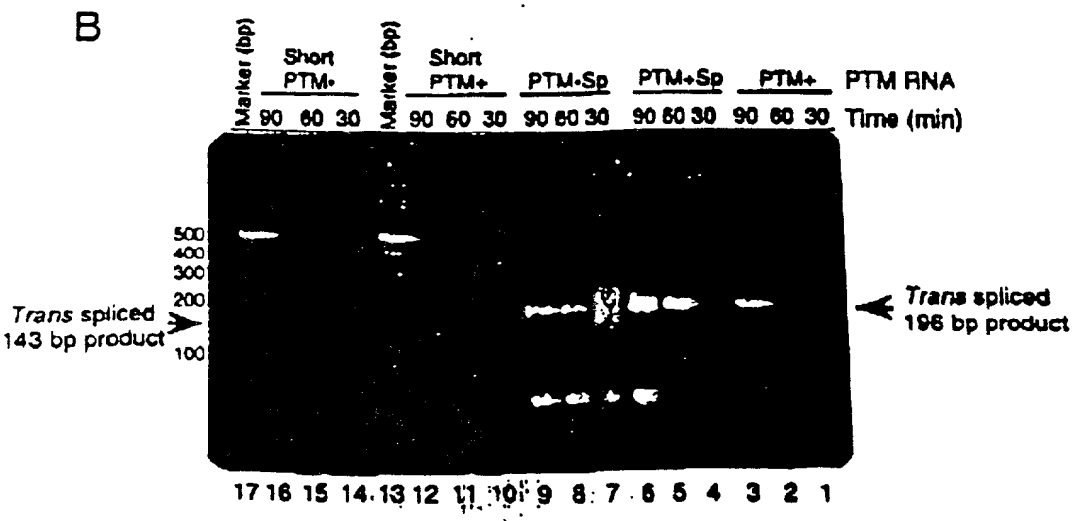


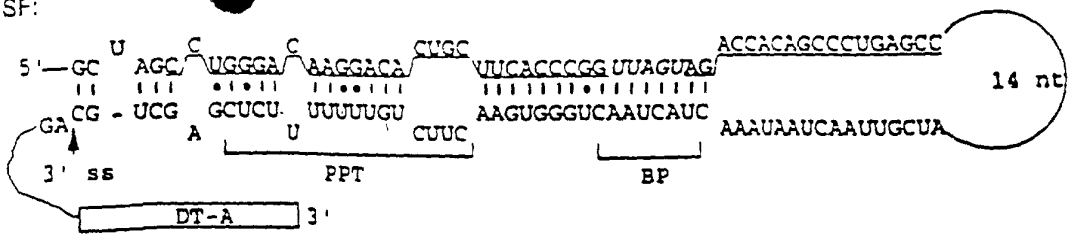
Figure 1B-C


A
PTM RNA
Time (min)
PTM+Sp
PTM-Sp
Py(-)AG(-)
BP(-)Py(-)AG(-)
Marker (bp)
0 60 0 60 0 60 0 60
Trans-spliced
466bp
product
600
500
400
300
1 2 3 4 5 6 7 8

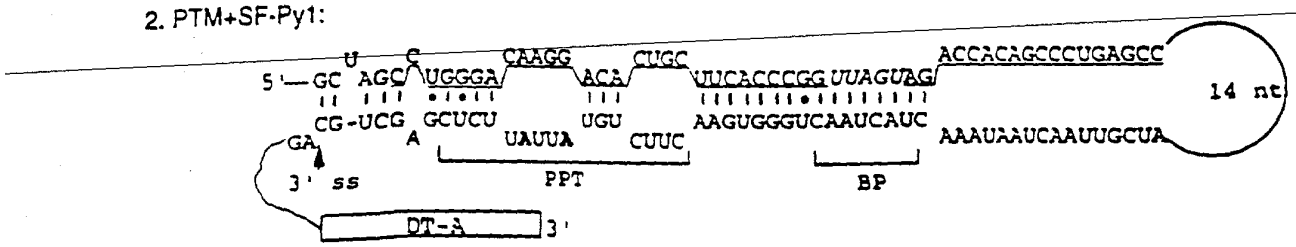

B
Marker (bp)
Short
PTM+
Marker (bp)
Short
PTM+
PTM-Sp
PTM+Sp
PTM+
PTM RNA
90 60 30 90 60 30 90 60 30 90 60 30 90 60 30
Time (min)
500
400
300
200
100
Trans spliced
143 bp product
Trans spliced
196 bp product
17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

Exon 1 of βHCG6
1st coding nucleotide of DT-A
C T A G

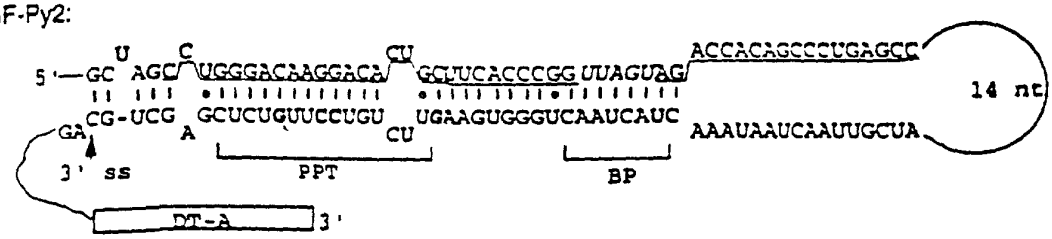
1. PTM+SF:



2. PTM+SF-Py1:



3. PTM+SF-Py2:



(B)

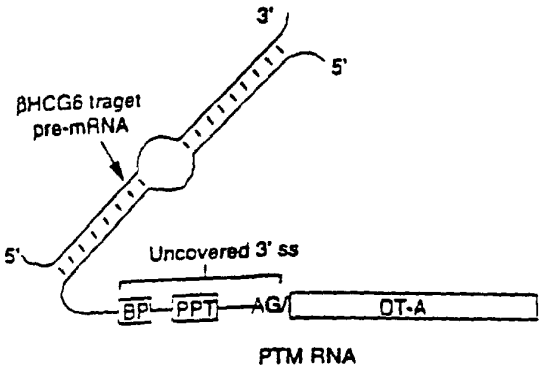


Figure 4A-B

(C)

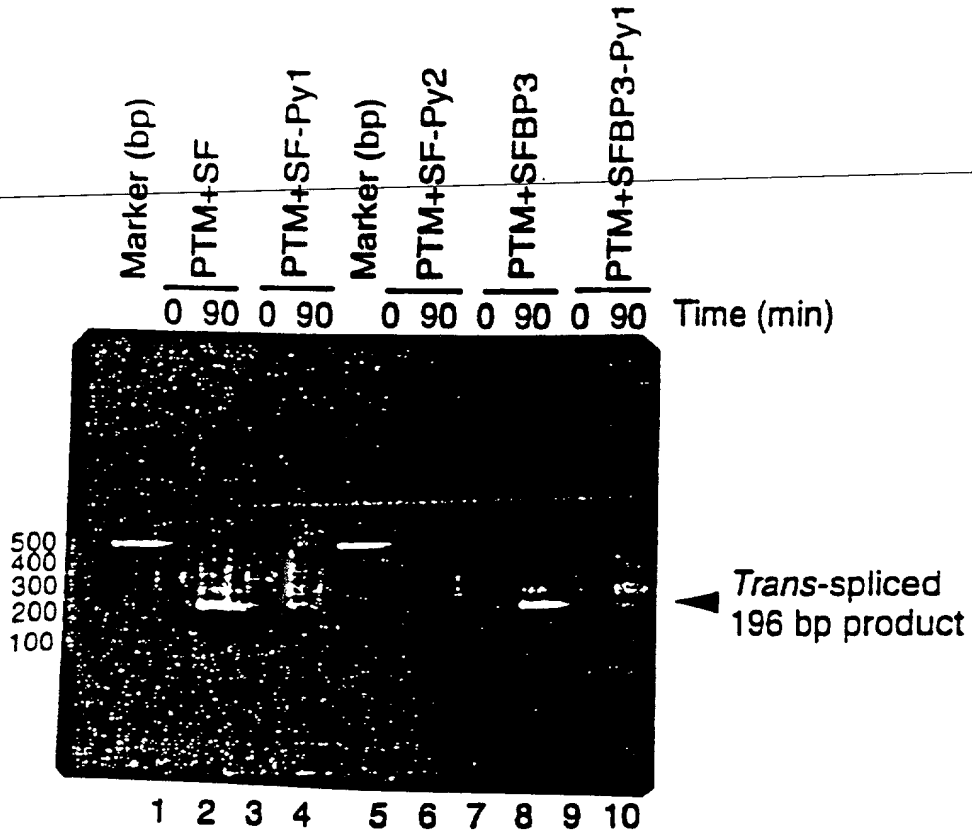

Marker (bp)
PTM+SF
PTM+SF-Py1
Marker (bp)
PTM+SF-Py2
PTM+SFBP3
PTM+SFBP3-Py1
0 90 0 90 0 90 0 90 0 90
Time (min)
500
400
300
200
100
Trans-spliced
196 bp product
1 2 3 4 5 6 7 8 9 10

Figure 4C

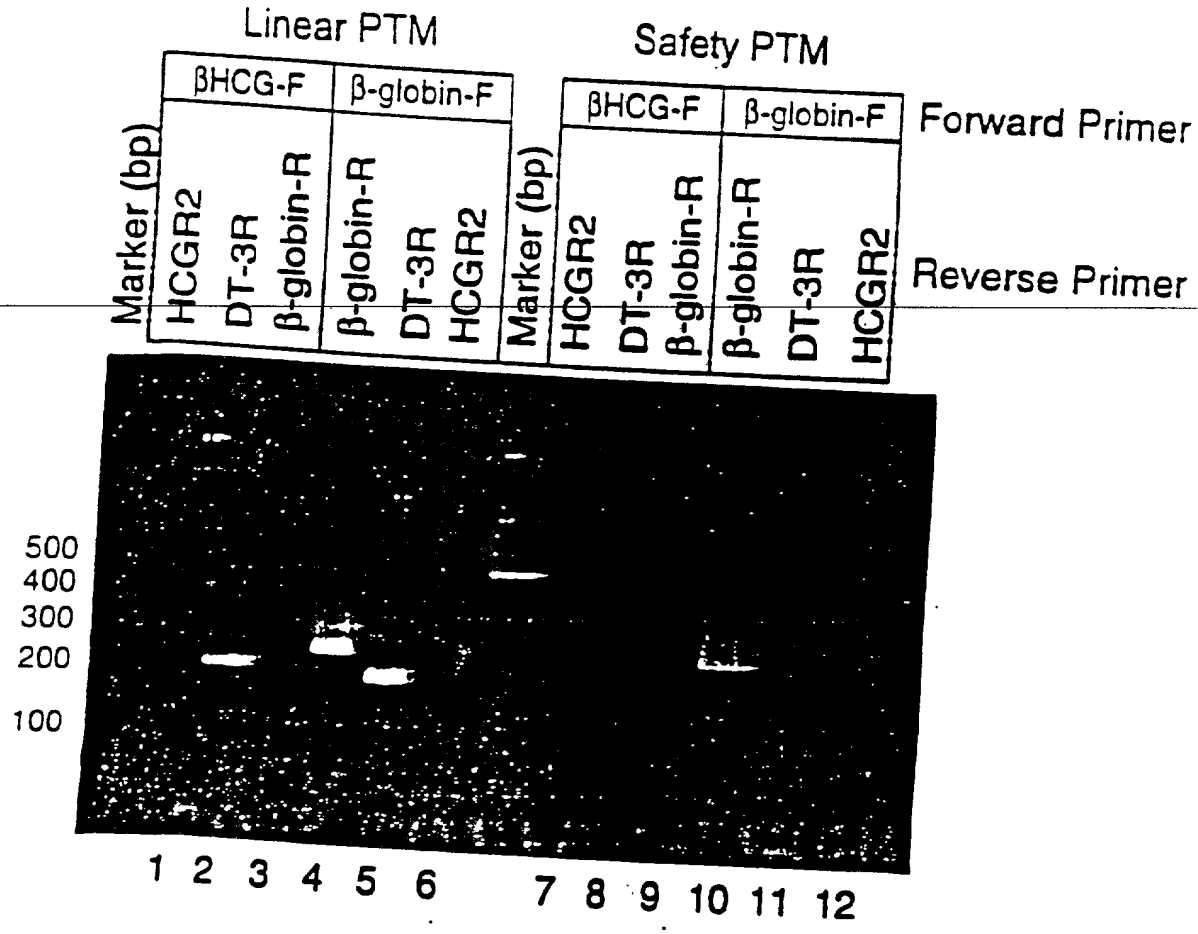

Linear PTM
Safety PTM
βHCG-F
β-globin-F
βHCG-F
β-globin-F
Forward Primer
Reverse Primer
Marker (bp)
HCGR2
DT-3R
β-globin-R
β-globin-R
DT-3R
HCGR2
Marker (bp)
HCGR2
DT-3R
β-globin-R
β-globin-R
DT-3R
HCGR2
500
400
300
200
100
1 2 3 4 5 6 7 8 9 10 11 12

Figure 5

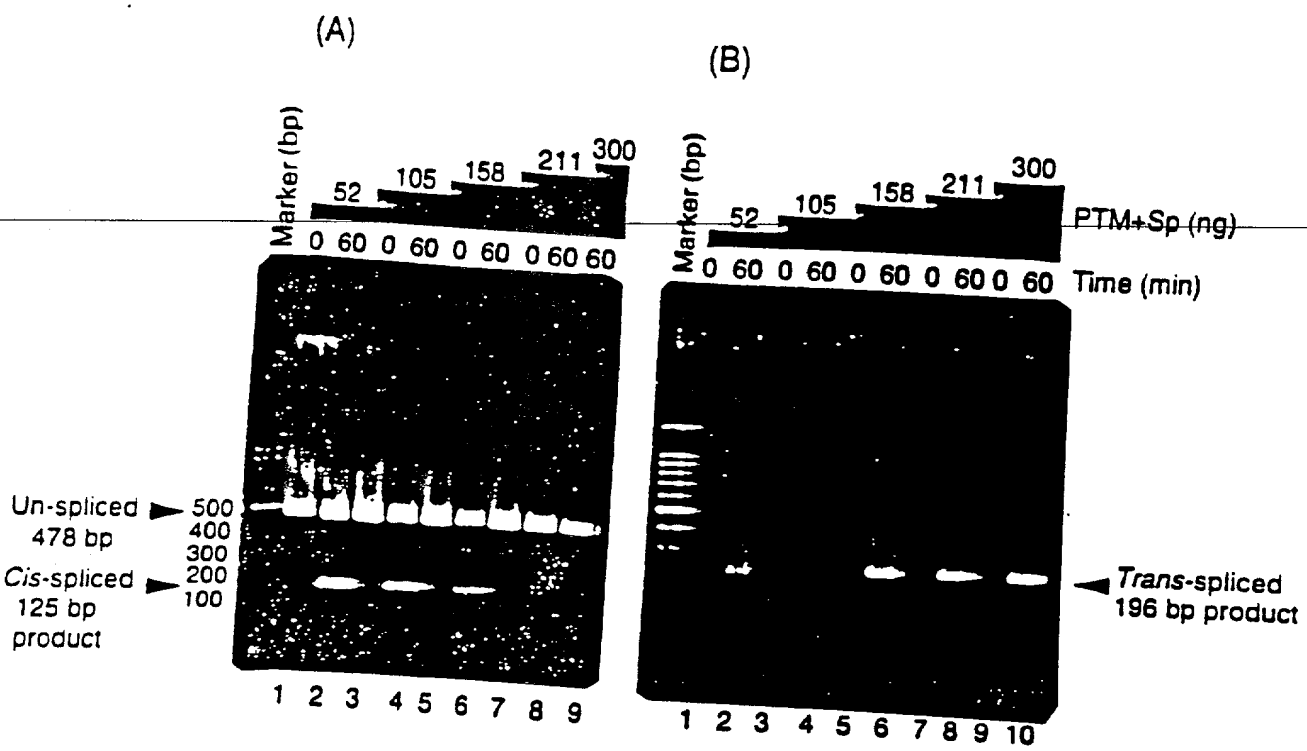
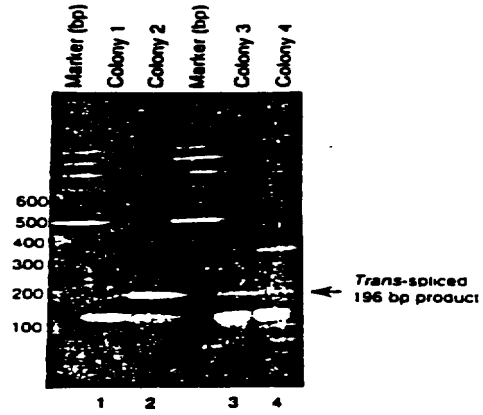

(A)
(B)
Marker (bp)
52
105
158
211
300
0 60 0 60 0 60 0 60 60
PTM+Sp (ng)
Time (min)
Un-spliced
478 bp
Cis-spliced
125 bp
product
500
400
300
200
100
Trans-spliced
196 bp product
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9 10

Figure 6

Figure 7



(B)

Exon 1 of βHCG6

5'-CAGGGGACGCACCAAGGATGGAGATGTTCCAG-GGCGCTGATGATGTTGTT

1st coding nucleotide of DT-A

GATTCTTCTTAAATCTTTTGTGATGGAAAACTTTTCTTCGTACCACGGGACTA

AACCTGGTTATGTAGATTCCATTCAAAAA-3'

Double Splicing Pre-therapeutic RNA

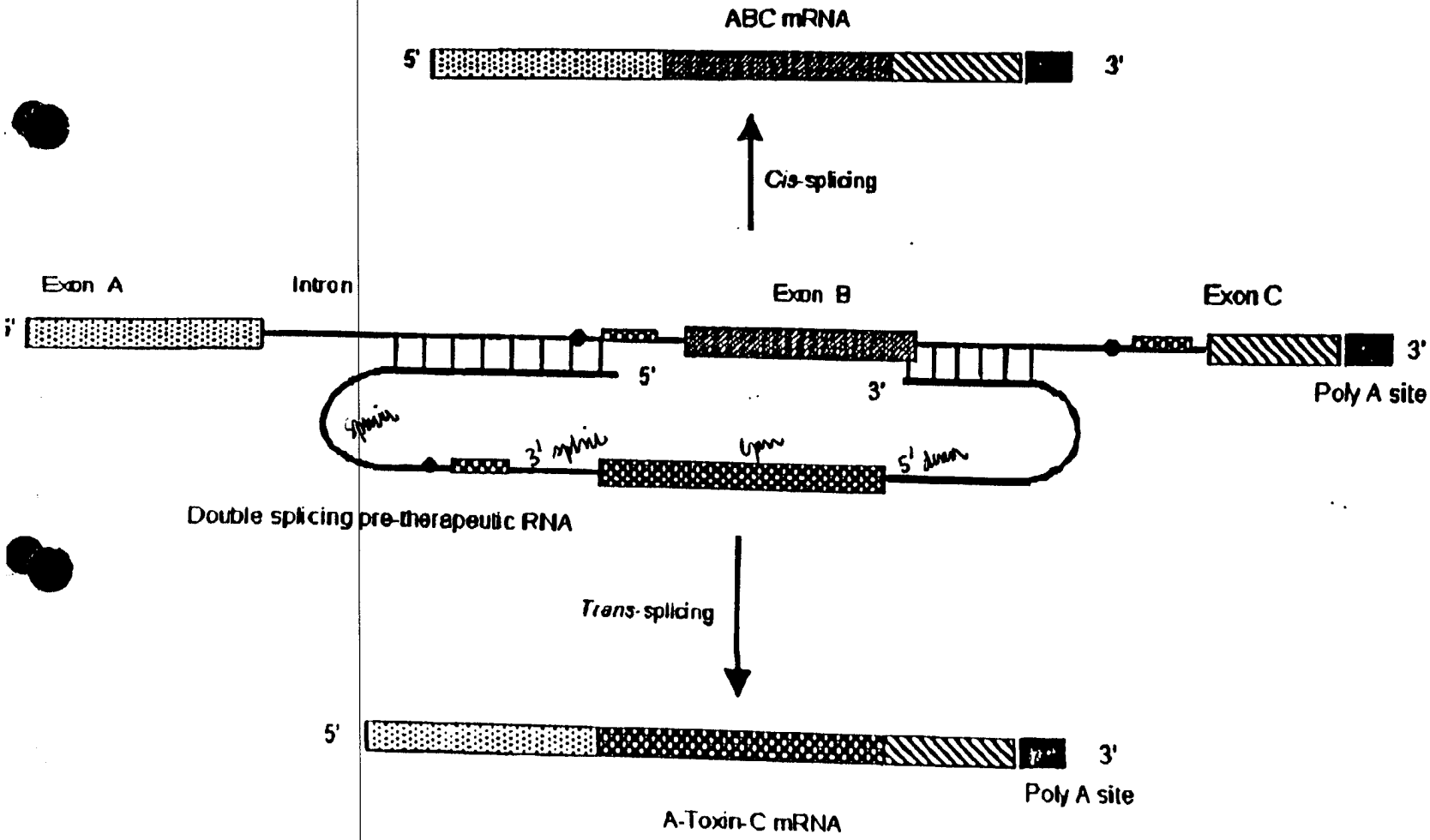


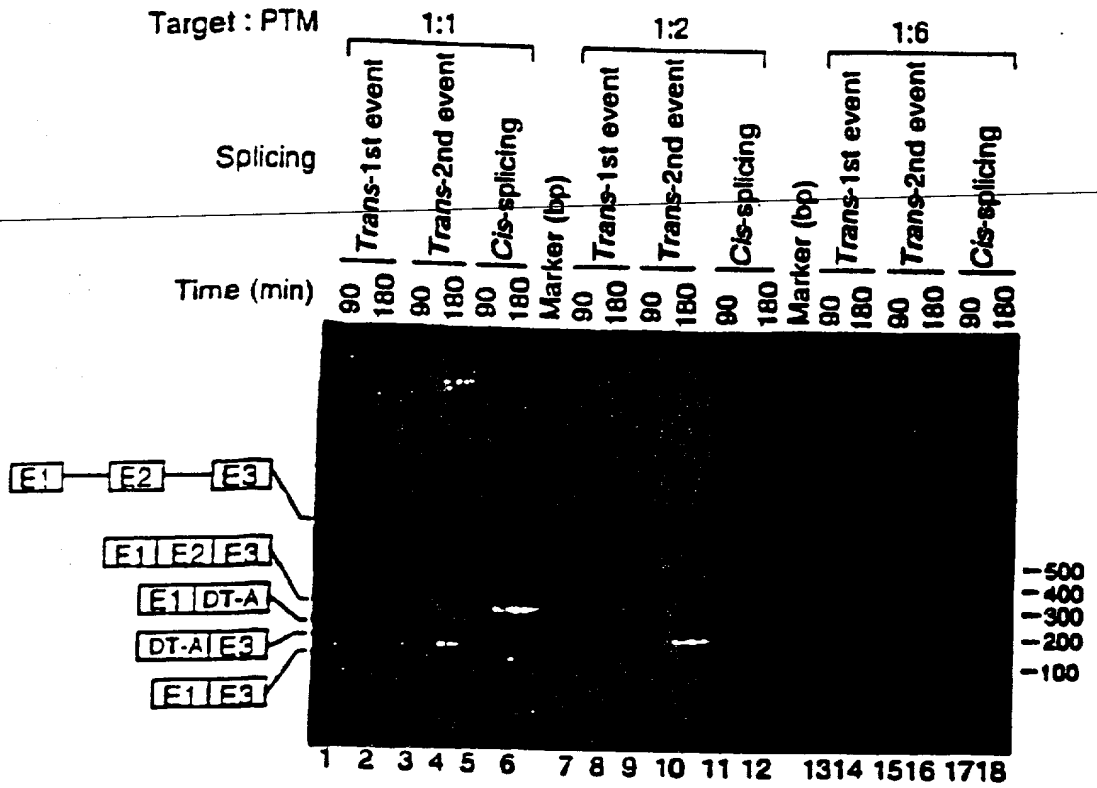
Figure 8A

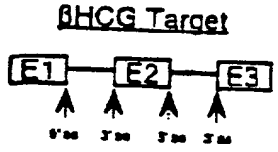
31304B-A

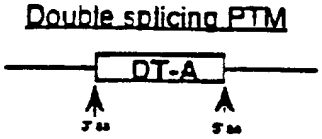
(Sheet 100f 66)

Sele[redacted]e *Trans*-splicing of a Double Splicing PTM

(3' ss of PTM to 5' ss target and, 5' ss of PTM to 3' ss of target)







Cis-spliced products

E1 E2 E3 = Normal *cis*-splicing (277bp)

E1 E3 = Exon skipping (110bp)

Trans-splicied products

E1 DT-A = 1st event, 196bp. *Trans*-splicing between 5' ss of target & 3' ss of PTM.

DT-A E3 = 2nd event, 161bp. *Trans*-splicing between 3' ss of target & 5' ss of PTM.

Figure 8B

31304B -A

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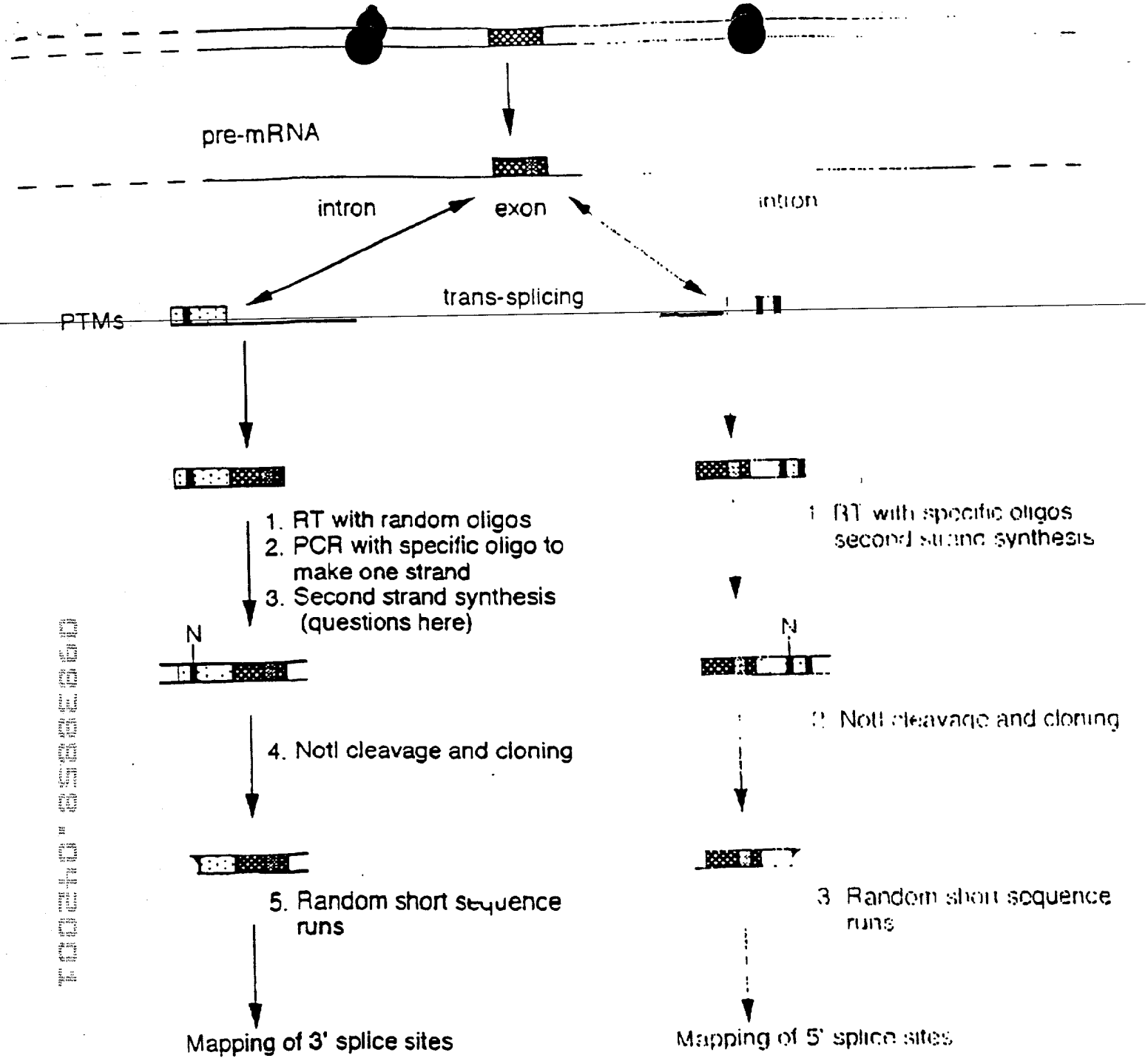
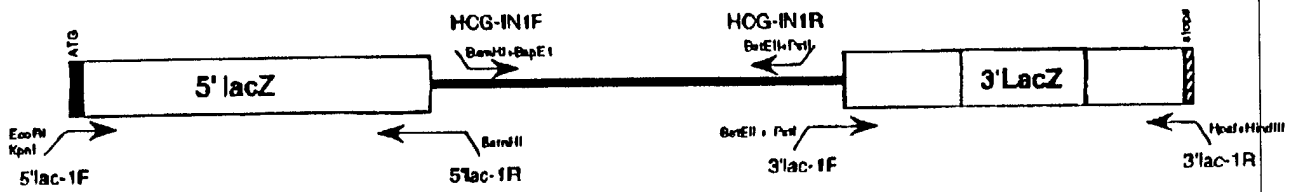

pre-mRNA
intron
exon
intron
trans-splicing
PTMs
1. RT with random oligos
2. PCR with specific oligo to make one strand
3. Second strand synthesis (questions here)
N
4. NotI cleavage and cloning
5. Random short sequence runs
Mapping of 3' splice sites
1. RT with specific oligos
second strand synthesis
N
2. NotI cleavage and cloning
3. Random short sequence runs
Mapping of 5' splice sites

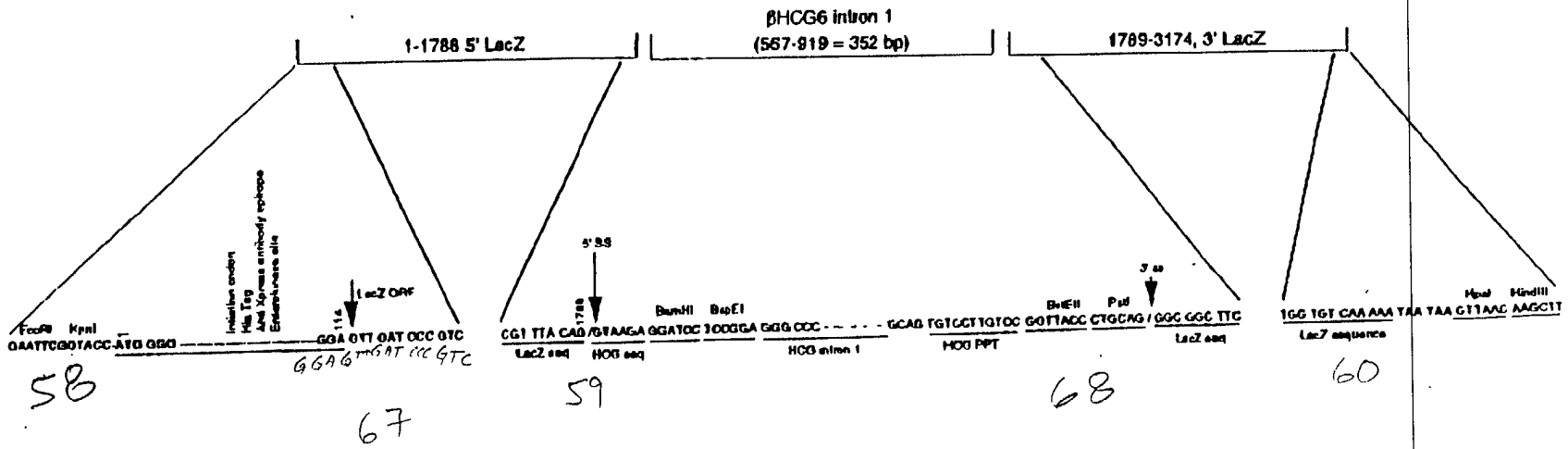
FIGURE 9

TDO Knock Out Vectors
LacZ Reporter Model Constructs

pc3·1 Lac-T1

Target 1:





PTMs

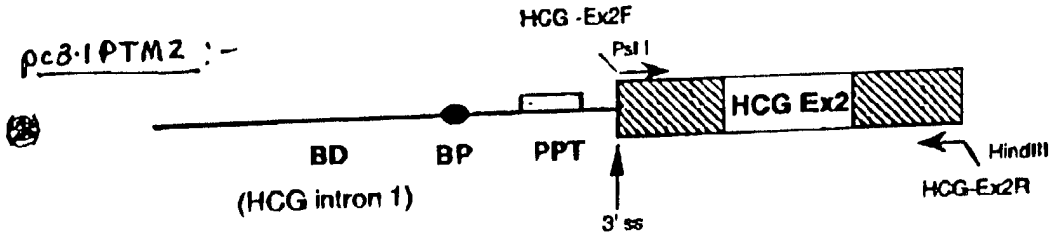


FIG. 10A

Restoration of β-Gal activity by SMaRT

(Spliceosome Mediated RNA*Trans*-splicing)

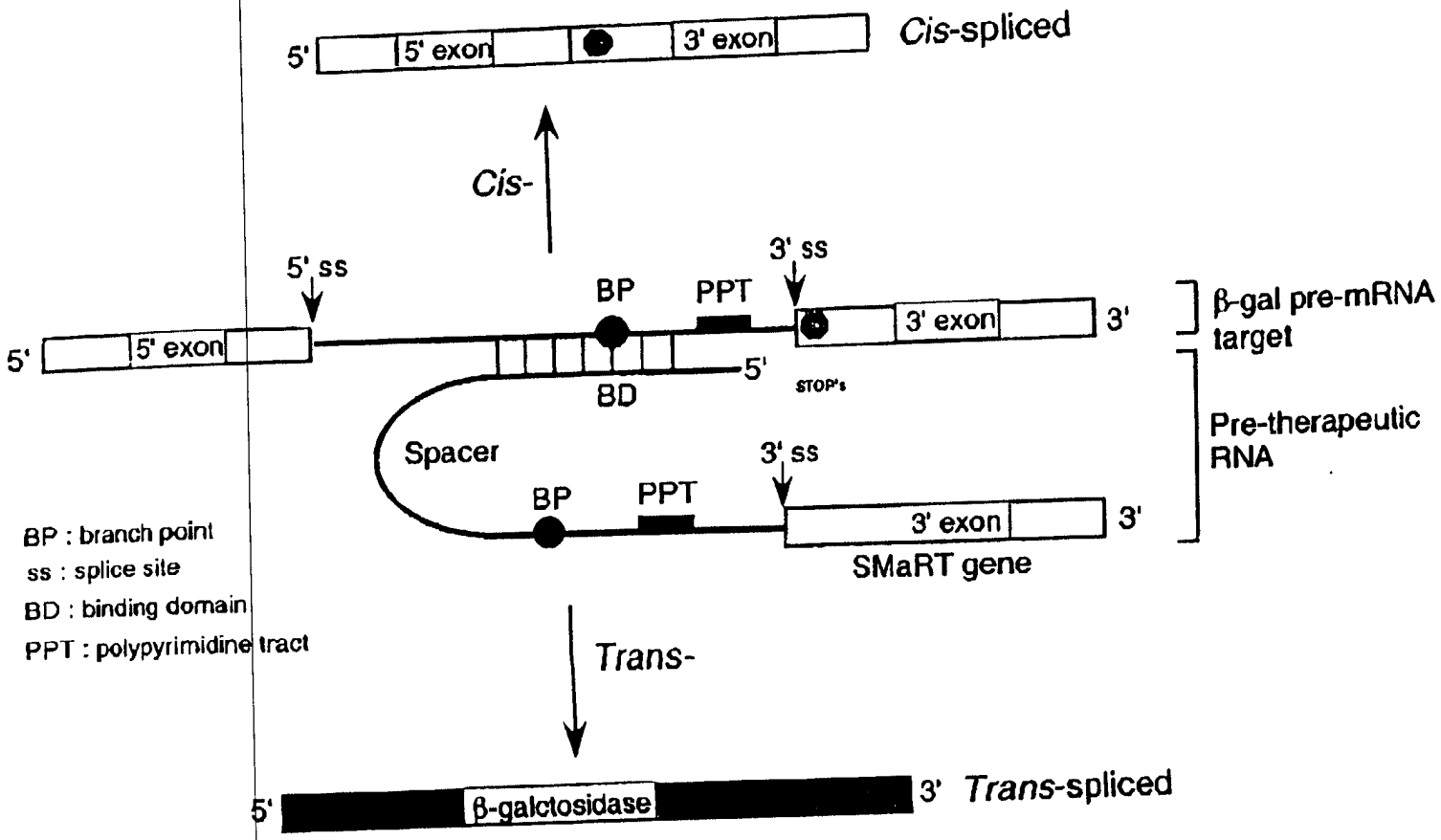


Figure 1D B

31304 B-A
(Mut 14 of 66)

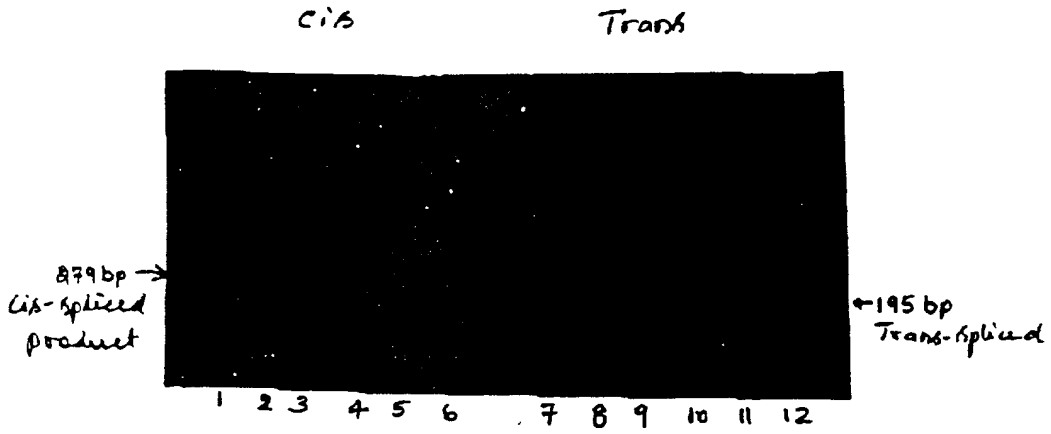

cis
Trans
879 bp
cis-spliced
product
195 bp
Trans-spliced
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 11A

Figure 11B

(Sheet 17 of 66)

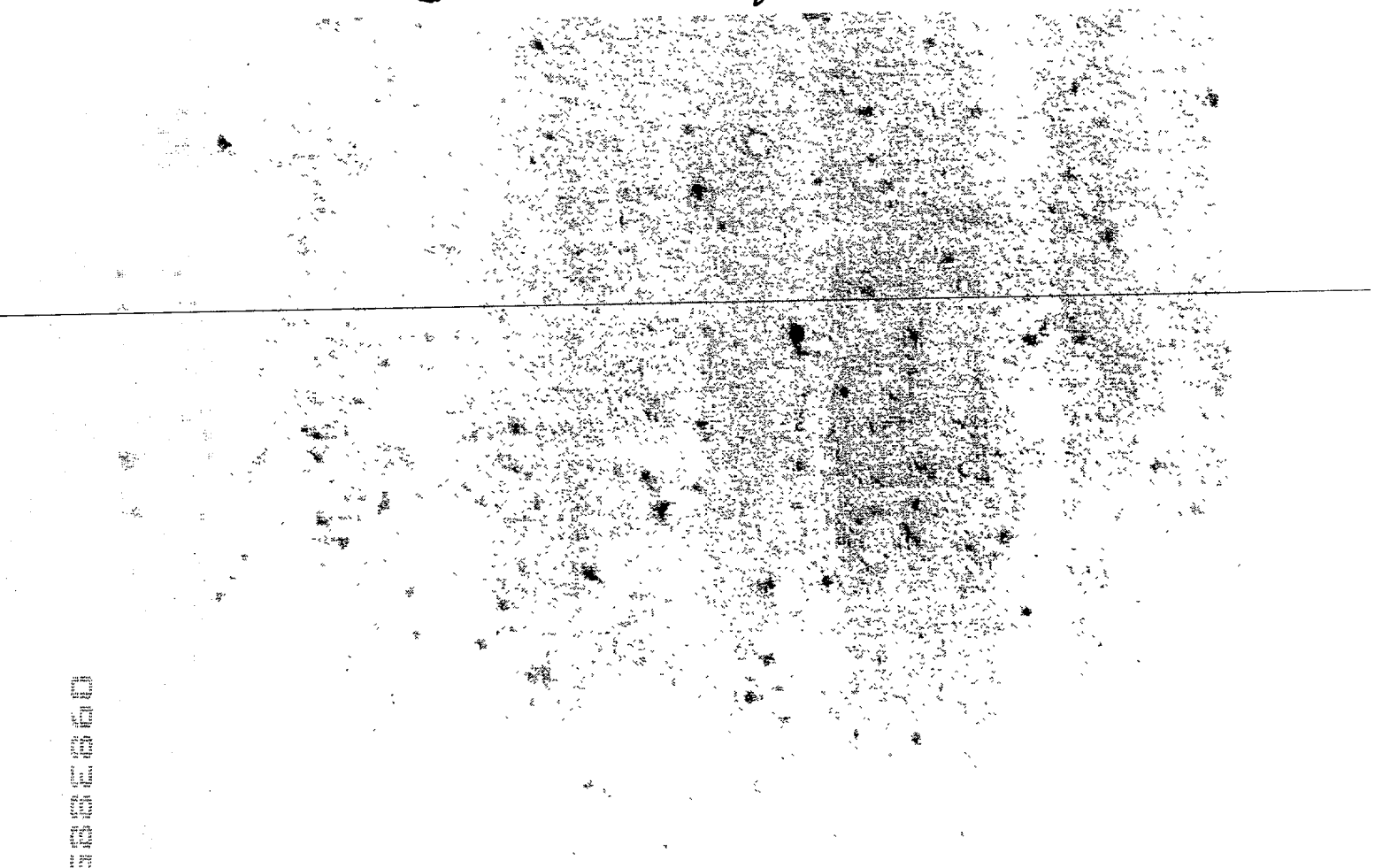


FIGURE IIC

Nucleotide Sequence Demonstrating that *Trans*-splicing is Accurate

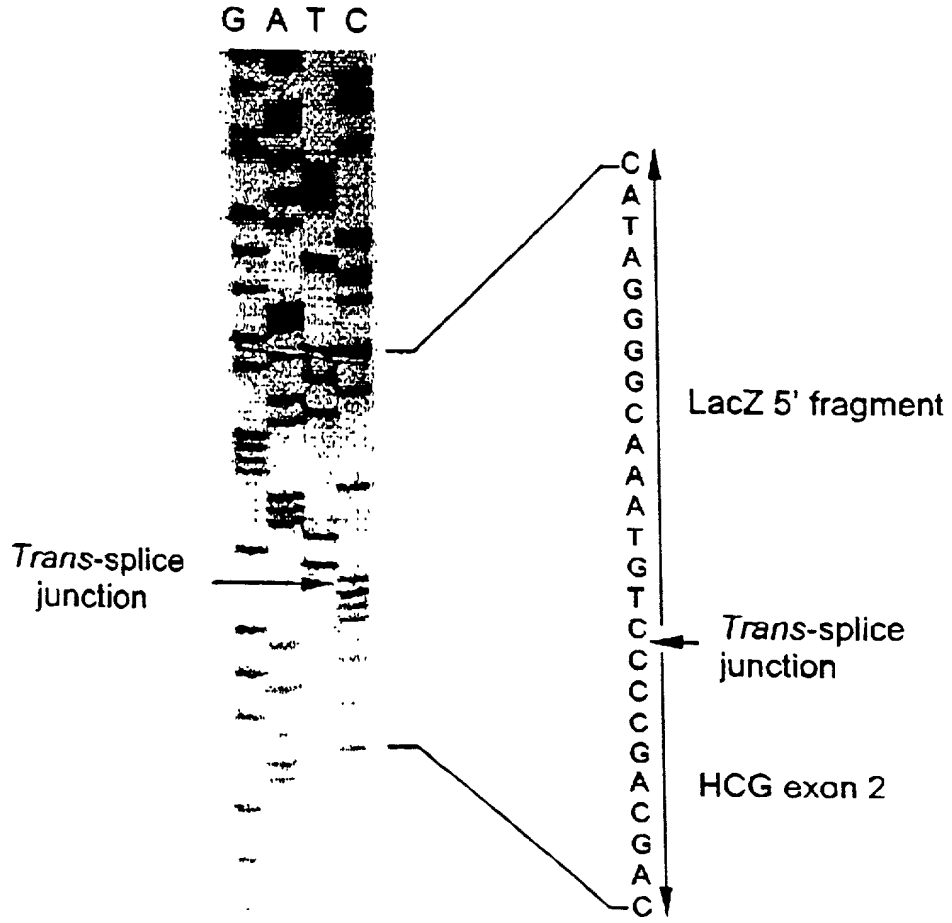


FIGURE 12A

31304-B-A

(Sheet 18 of 66)

(1). Nucleotide sequences of the *cis*-spliced product (285 bp) :

BioLac-TR1
GGCTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCACGCGATGGGTAACAGTCTTG

Splice junction
GCGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCCCGTTTACAG/GGCGGCTTCGTCTAATAATG

GGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGATTT

Lac-TR2
TGGCGATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGGCGACCGCACGCCGCATCCAG

(2) Nucleotide sequences of the trans-spliced product (195 bp)

BioLac-TR1
GGCTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCACGCGATGGGTAACAGTCTTGG

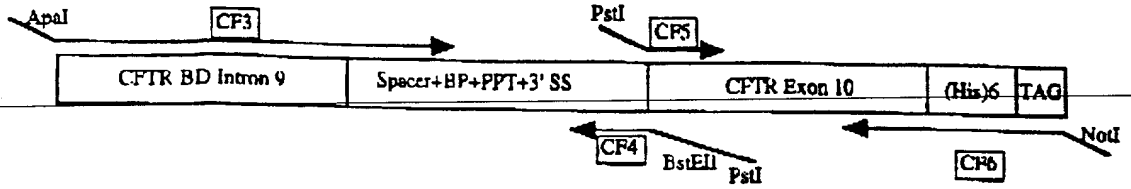
Splice junction
CGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCCCGTTTACAG/GGGCTGCTGCTGTTGCTGCTGCT

HCGR2
GAGCATGGGCGGGACATGGGCATCCAAGGAGCCACTTCGGCCACGGTGCCG

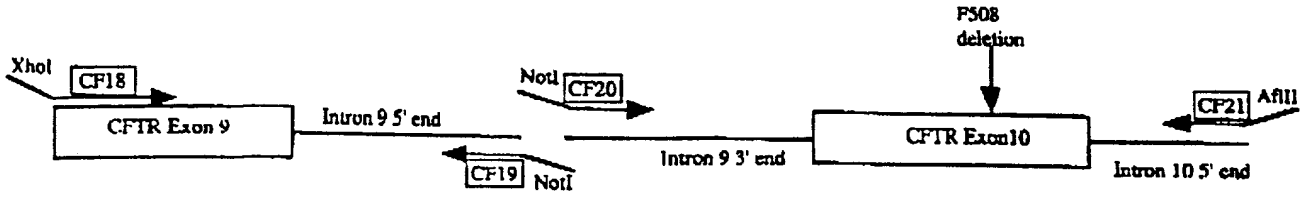
Figure 12 B

31304-B-A
(Shut 19 of 66)

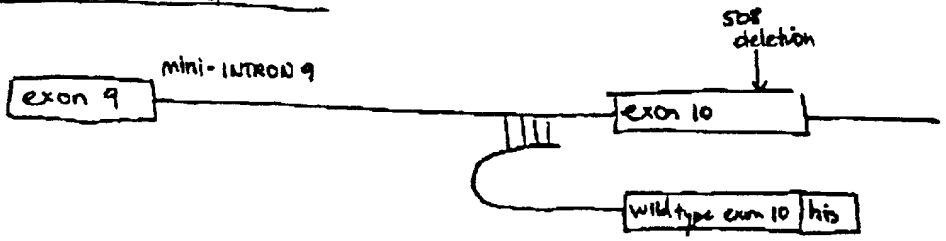
CFTR Pre-therapeutic molecule (PTM or "bullet")



CFTR mini-gene target - Construction



TRANS-SPLICING Repair



Binding of PTM to TARGET

↓ splicing

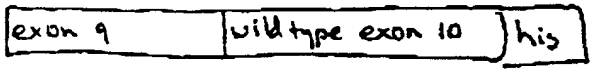
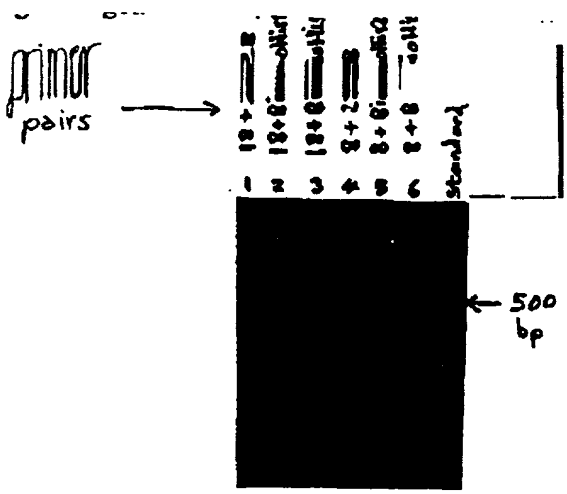


Figure 13

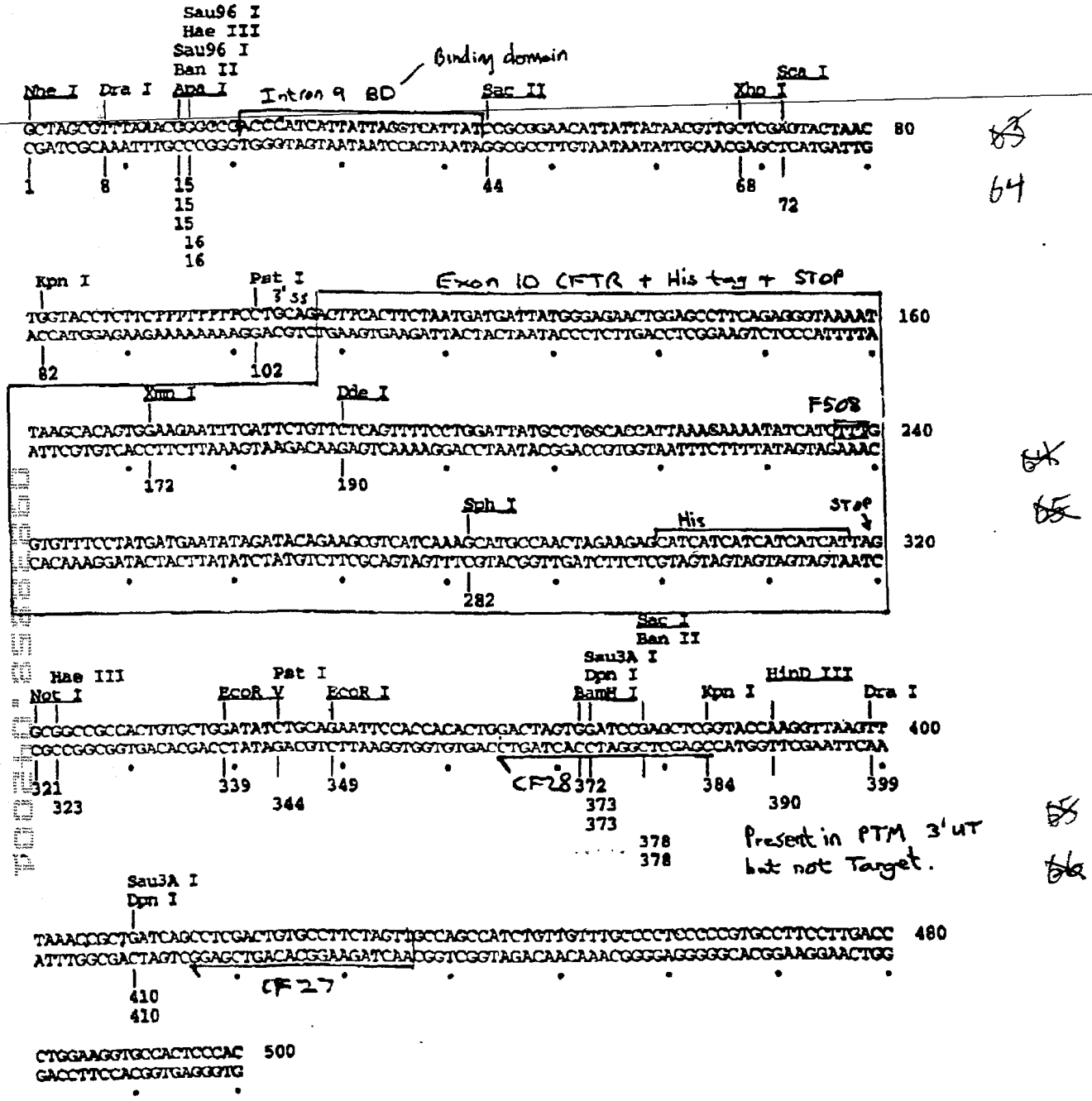
31304-B-A
(Shut 2004 66)

Figure 14



DNA sequence 500 b.p. GCTAGCGTTTAA ... TGCCACTCCCAC linear

Positions of Restriction Endonucleases sites (unique sites underlined)



Restriction Endonucleases site usage

Acc I	-	EcoR I	1	Nde I	-	Sau96 I	2
Apa I	1	EcoR V	1	Nhe I	1	Sca I	1
ApaL I	-	Hae II	-	Not I	1	Sma I	-
Avr II	-	Hae III	2	PflM I	-	Sph I	1
BamH I	1	HinC II	-	Pst I	2	Spl I	-
Ban II	2	HinD III	1	Pvu I	-	Ssp I	
Bbe I	-	Hinf I	-	Pvu II	-	Stu I	

31304-A-B
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EXPERIMENT 12

Repair of an exogenously supplied CFTR target molecule carrying an F508 deletion in exon 10.

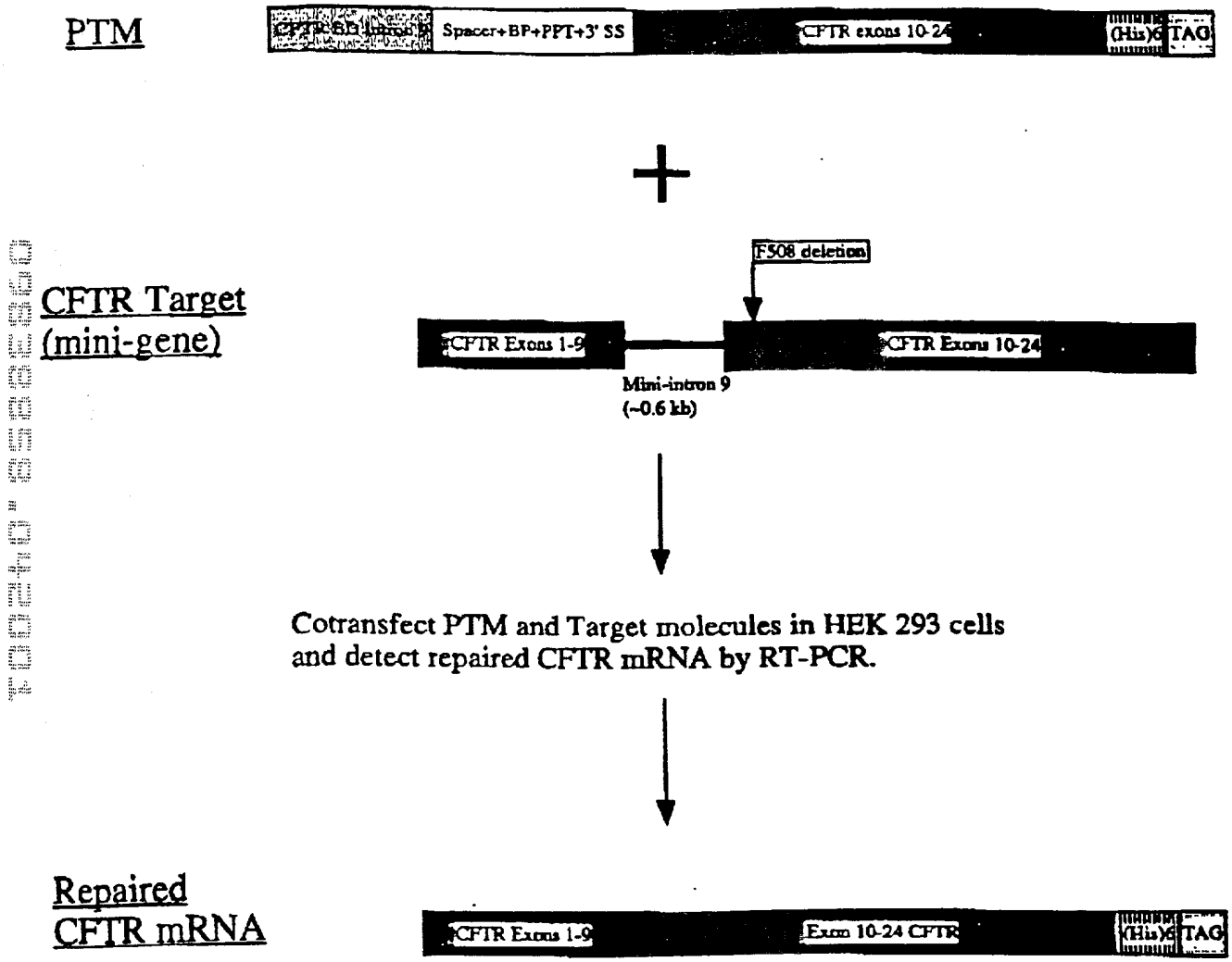

Figure 16
31304-A-B
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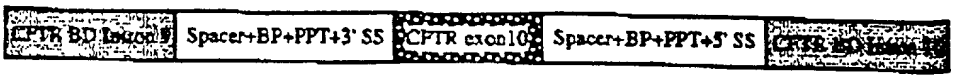
EXPERIMENT 3

Repair of endogenous CFTR transcripts by exon 10 invasion using a double splicing PTM

Double Splicing PTM



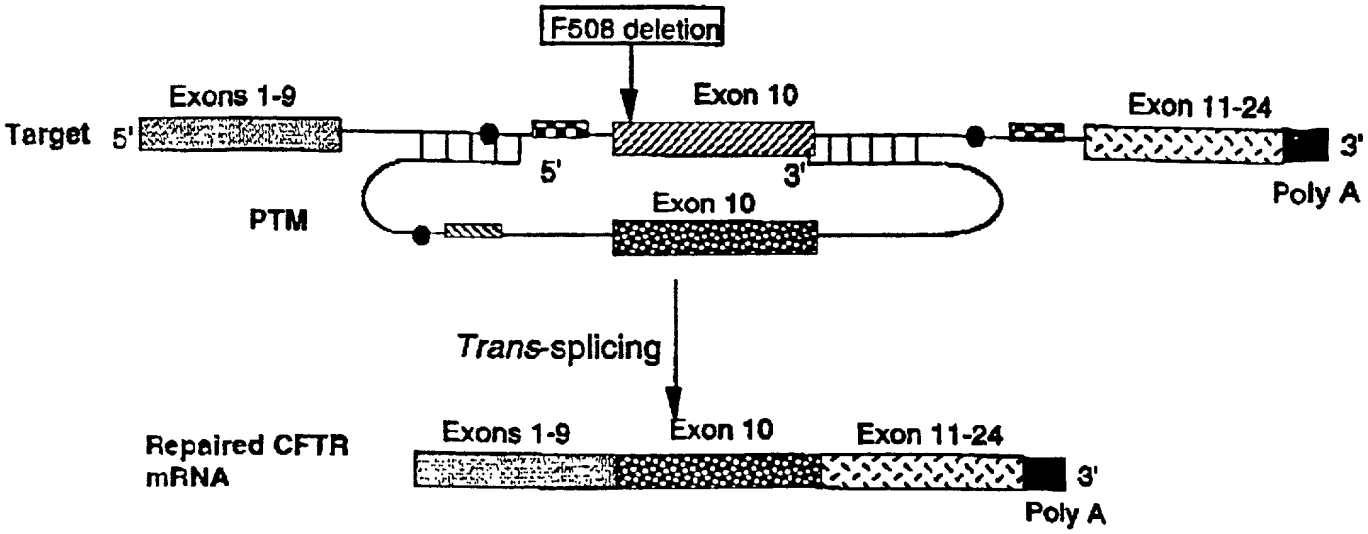


Figure 17

31304 B-A

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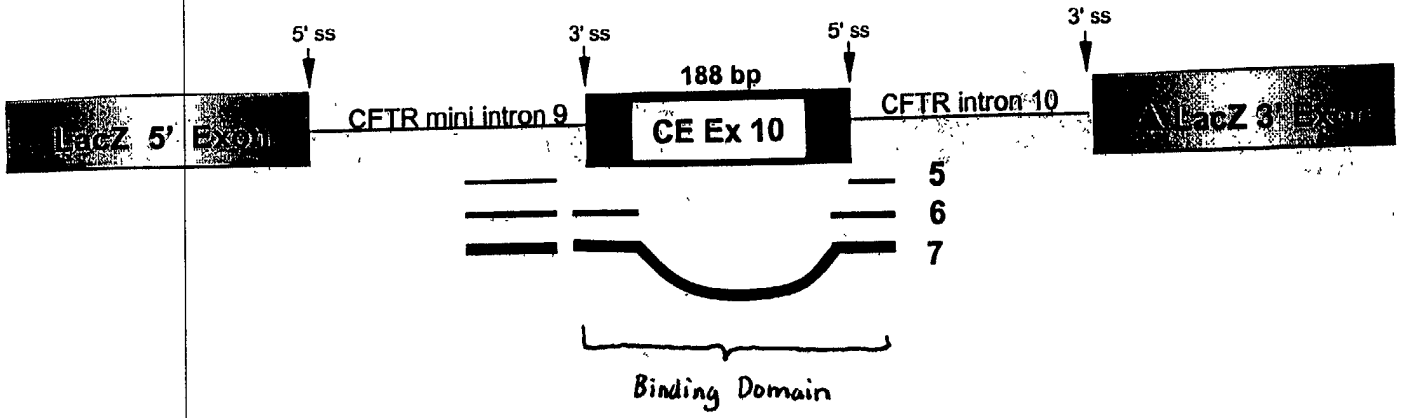
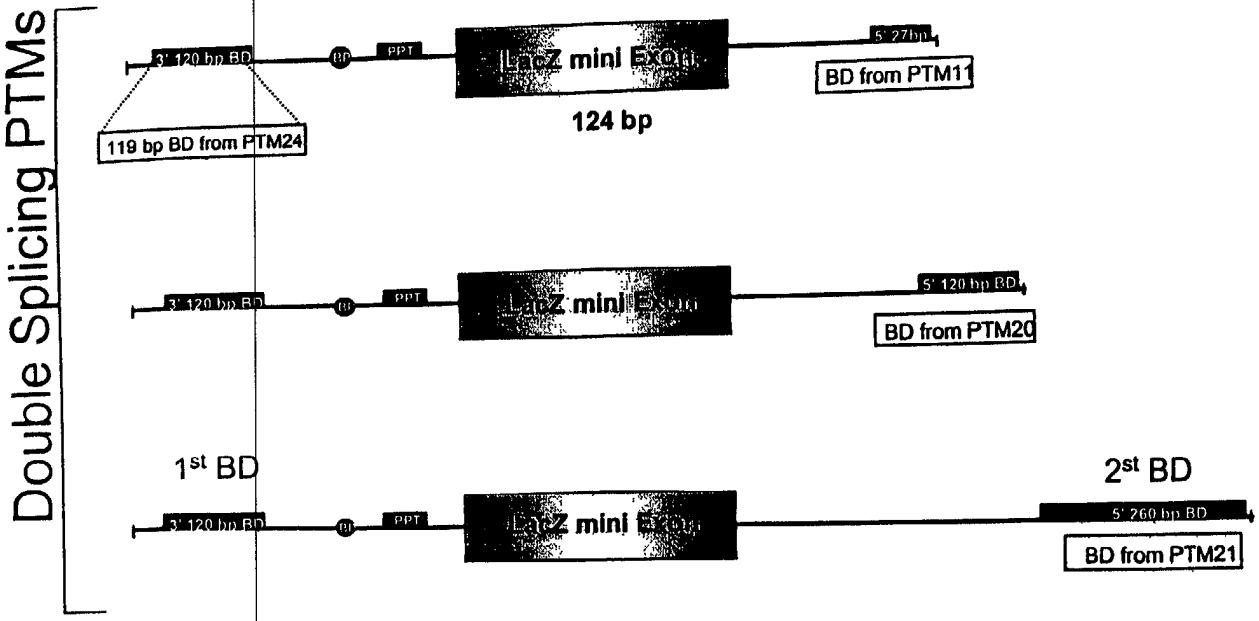
Double *Trans*-splicing Specific Target



Target DSCFT1-6

Figure 18

Double *Trans*-splicing PTMs



DSPTM-5

PTM with 27 bp BD & masks 5' single splice site

DSPTM-6

PTM with 120 bp BD & masks both 5' & 3' splice sites

DSPTM-7

PTM with 260 bp BD masking both the ss & the entire CFTR Ex10

Figure 19

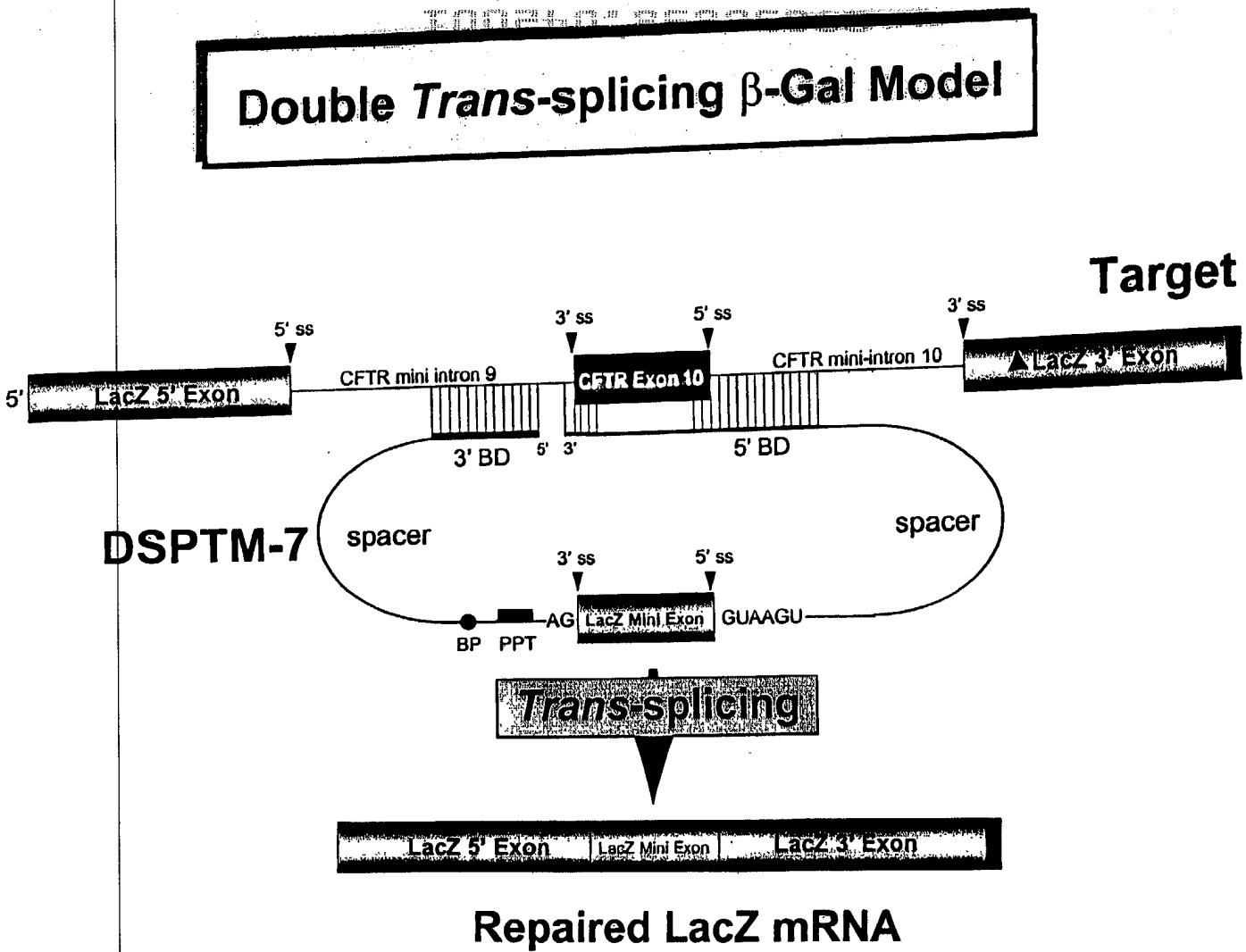
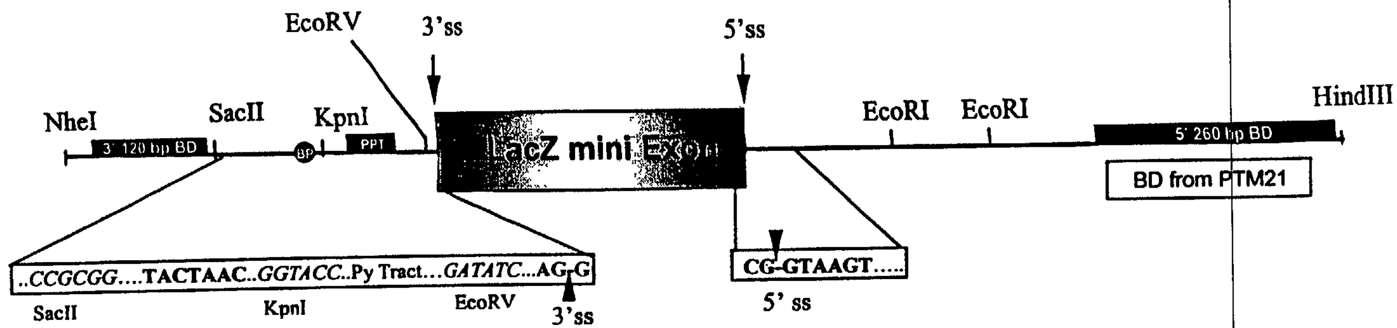

Double Trans-splicing β-Gal Model
Target
5' ss
3' ss
5' ss
3' ss
5'
LacZ 5' Exon
CFTR mini intron 9
CFTR Exon 10
CFTR mini-intron 10
LacZ 3' Exon
3' BD
5'
3'
5' BD
DSPTM-7
spacer
spacer
3' ss
5' ss
BP
PPT
AG
LacZ Mini Exon
GUAAGU
Trans-splicing
LacZ 5' Exon
LacZ Mini Exon
LacZ 3' Exon
Repaired LacZ mRNA

Figure 20

Important Structural Elements of DSPTM-7: (Double splicing PTM with all the necessary splice elements i.e. has both 3' and 5' functional splice sites and the binding domains)



(1) 3' BD (120 BP) : GATTCACTTGCTCCAATTATCATCCTAAGCAGAAGTGTATATTCTTATTTGTAAAGATTCTATTAACTCATTTGATTC AAAATATTTAAAATACTTCCTGTTTCATACTCTGCTATGCAC

(2) Spacer sequences (24 bp): AACATTATTATAACGTTGCTCGAA

(3) Branch point, pyrimidine tract and acceptor splice site: TACTAAC T *GGTACC* TCTTCTTTTTTTTTTT *GATATC* CTGCAG GGC GGC

(BP: TACTAAC; Kpn I: GGTACC; PPT: TCTTCTTTTTTTTTTT; EcoRV: GATATC; 3' ss ↓ before GGC GGC (LacZ mini exon))

(4) 5' donor site and 2nd spacer sequence: TGA ACG *GTAAGT* GTTATCACCGATATGTGTCTAACCTGATTCGGGCCTTCGATACG

CTAAGATCCACCGG

(LacZ mini exon: TGA ACG; 5' ss ↓ before GTAAGT)

(5) 5' BD (260 BP) : TCAAAAAGTTTTCACATAATTTCTTACCTCTTCTT*GAATTC*ATGCTTTGATGACGCTTCTGTATCTATATTCATCATTGGAA ACACCAATGATTTTTCTTTAATGGTGCCTGGCATAATCCTGGAAAACTGATAACACAATGAAATTCTTCCACTGTGCTTAA AAAAACCCTCT*GAATTC*TCCATTTCTCCCATAATCATCATTACAACTGAACTCTGGAAATAAAACCCATCATTATTAACTCA TTATCAAATCACGC

4

Figure 21

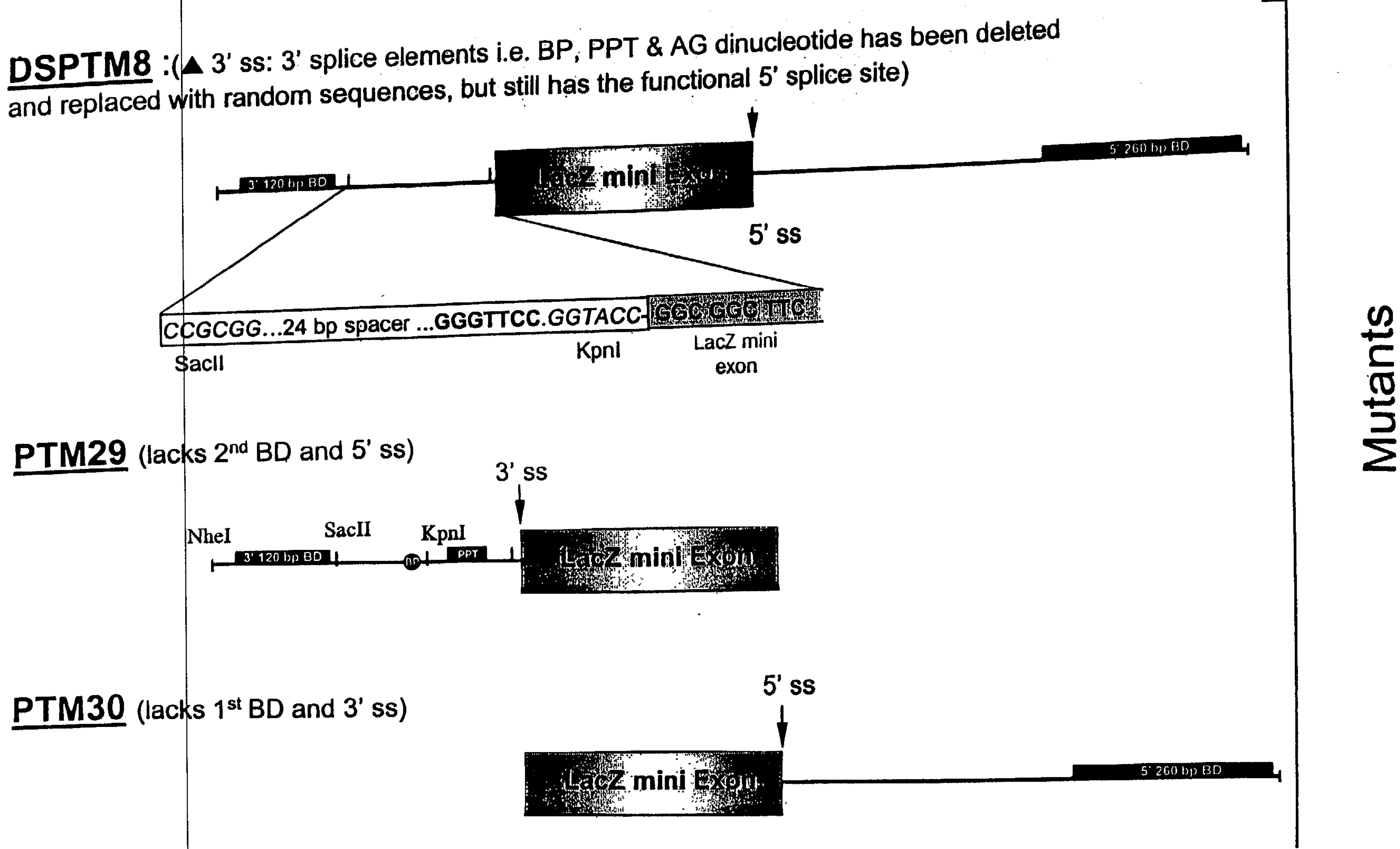

DSPTM8 :(▲ 3' ss: 3' splice elements i.e. BP, PPT & AG dinucleotide has been deleted and replaced with random sequences, but still has the functional 5' splice site)
3' 120 bp BD
LacZ mini Exon
5' 260 bp BD
5' ss
CCGCGG...24 bp spacer ...GGGTTCC.GGTACC-GGC GGC TTC
SacII
KpnI
LacZ mini exon
PTM29 (lacks 2nd BD and 5' ss)
3' ss
NheI
SacII
KpnI
3' 120 bp BD
BP
PPT
LacZ mini Exon
PTM30 (lacks 1st BD and 3' ss)
5' ss
LacZ mini Exon
5' 260 bp BD
Mutants

Figure 22

Accuracy of Double *Trans*-splicing Reaction

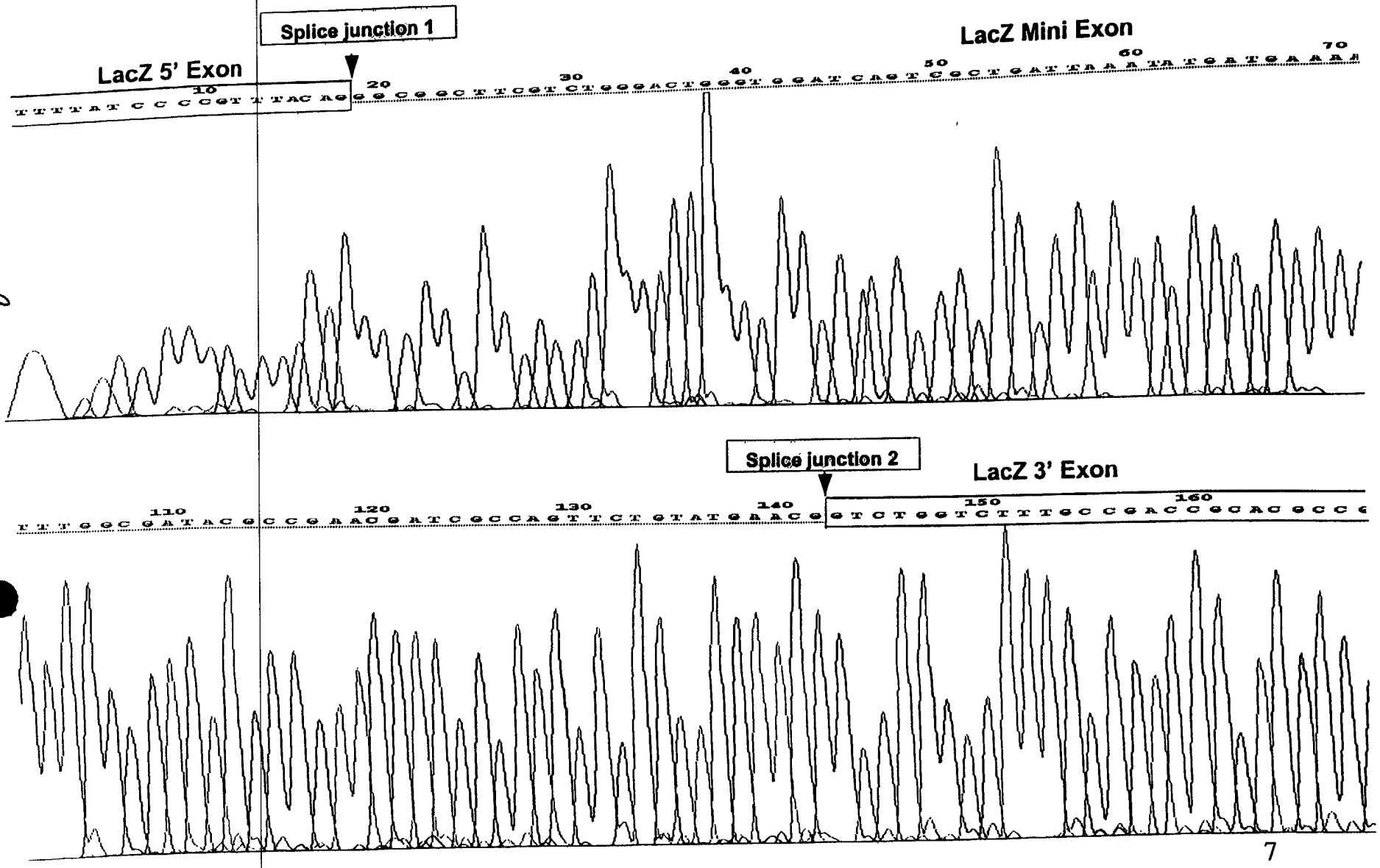
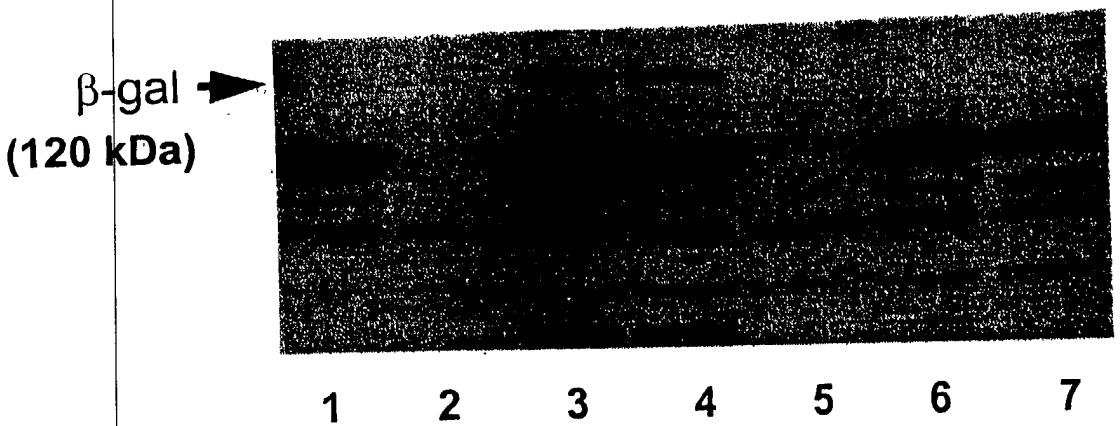


Figure 23

Double *Trans*-splicing Produces Full-length Protein



Lane 1: DSCFT1.6 Target alone	25 μg
Lane 2: DSPTM7	25 μg
Lane 3 Target + PTM #6	25 μg
Lane 4: Target + PTM #9	25 μg
Lane 5: Delta 3' splice mutant alone	25 μg
Lane 6: Target + Delta 3' ss	25 μg
Lane 7: Target+PTM29+30 (mutants)	25 μg

Figure 24

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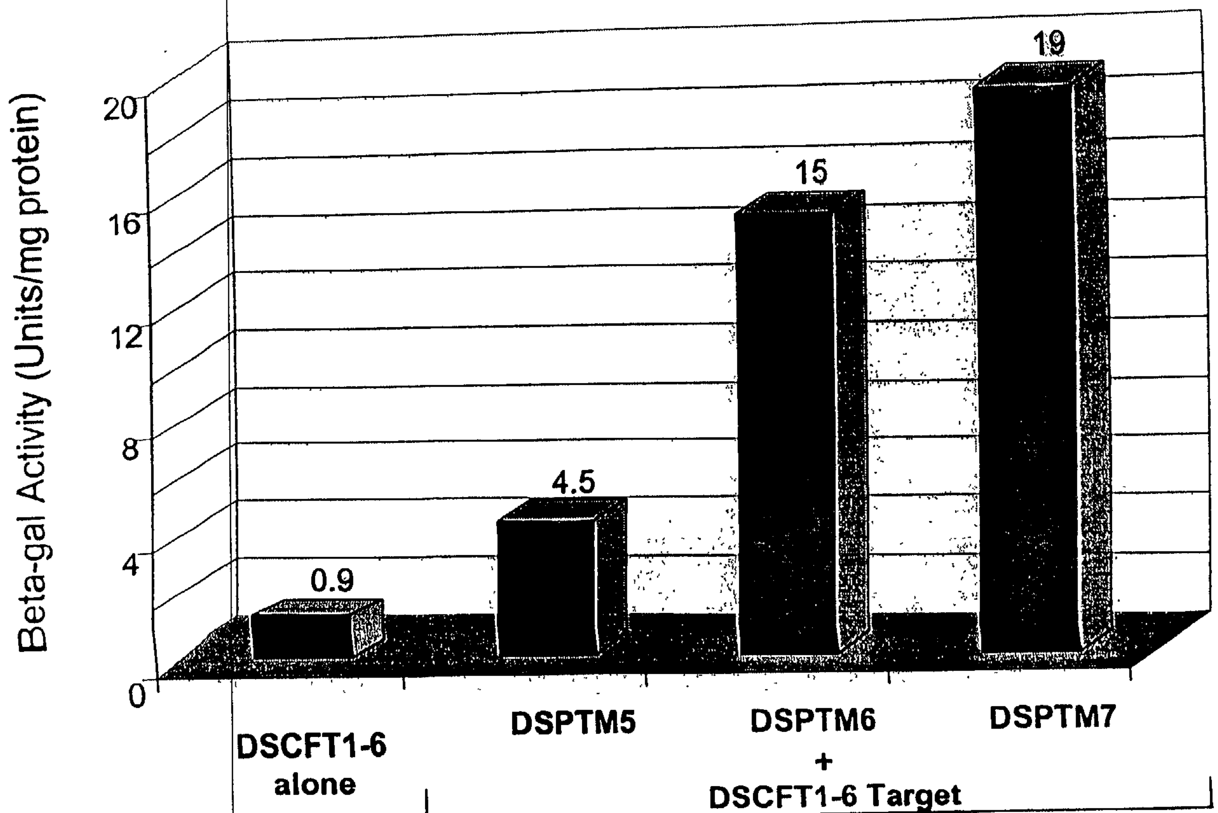
Restoration of β-Gal Function by Double *Trans*-splicing

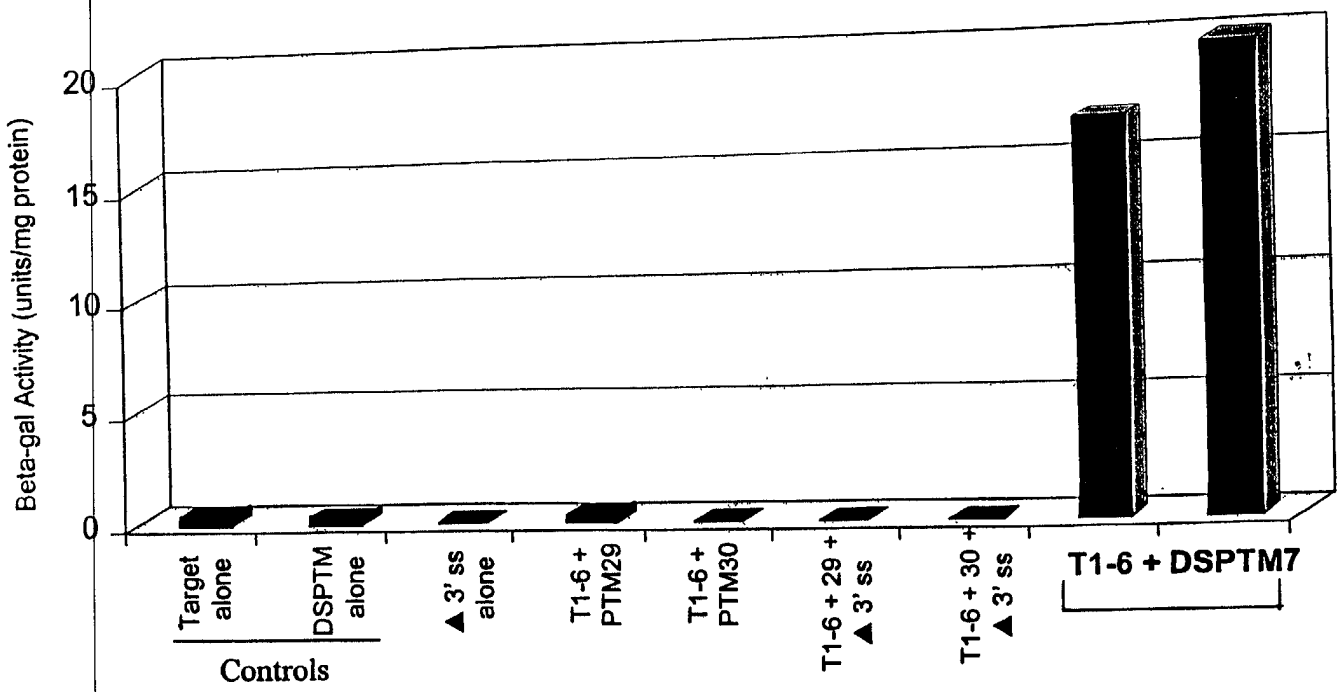


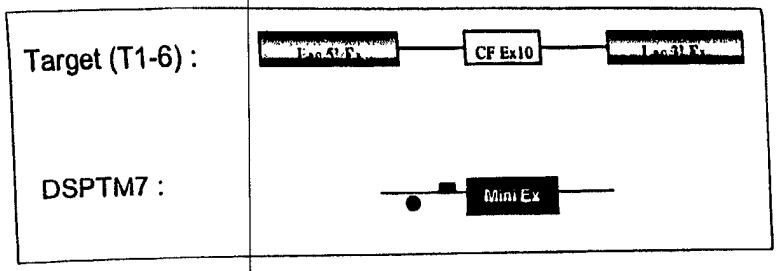
Beta-gal Activity above the Background level

DSPTM5:	5 fold
DSPTM6:	17 fold
DSPTM7:	21 fold

Figure 25

Restoration of β-gal activity is due to double RNA *trans*-splicing events





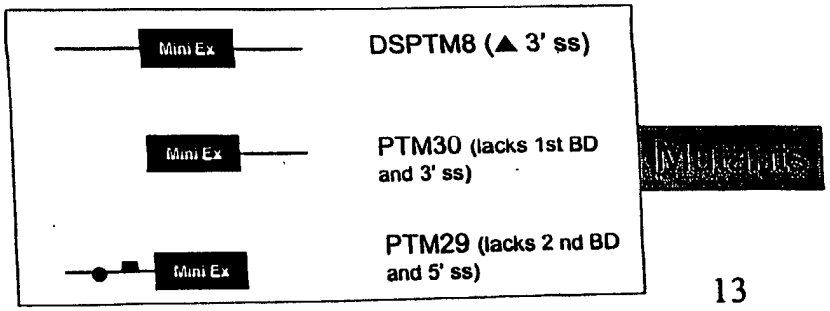
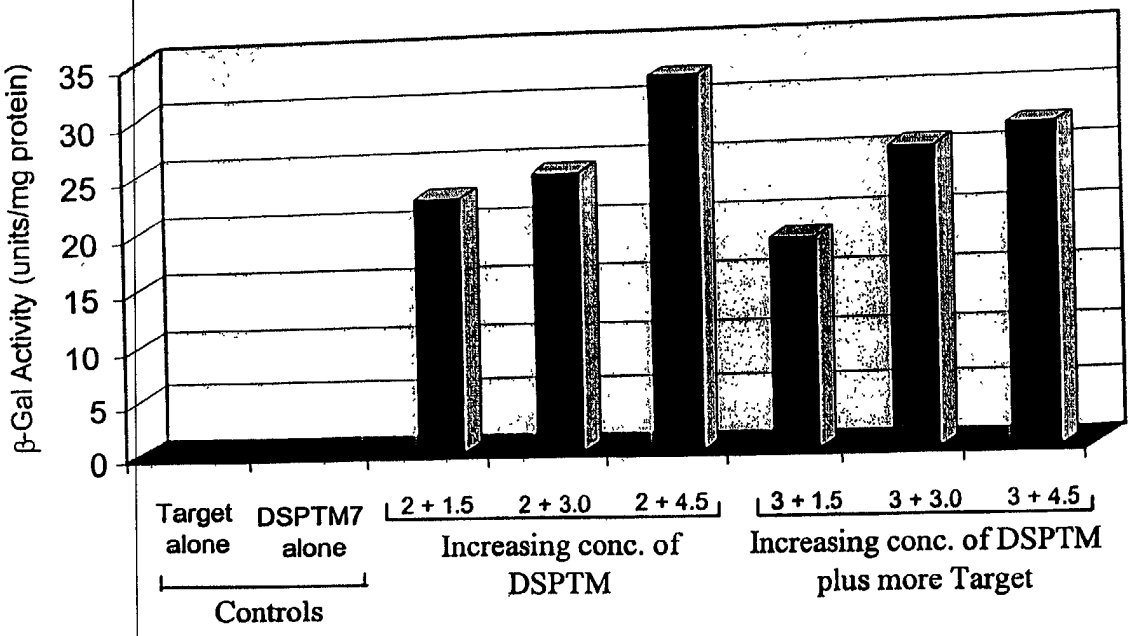


Figure 26

Double *Trans*-splicing: Titration of Target & PTM



The current level of beta-gal activity due to double *trans*-splicing is ~ 1-1.5% of the best single splice model (3' exon replacement)

Figure 27

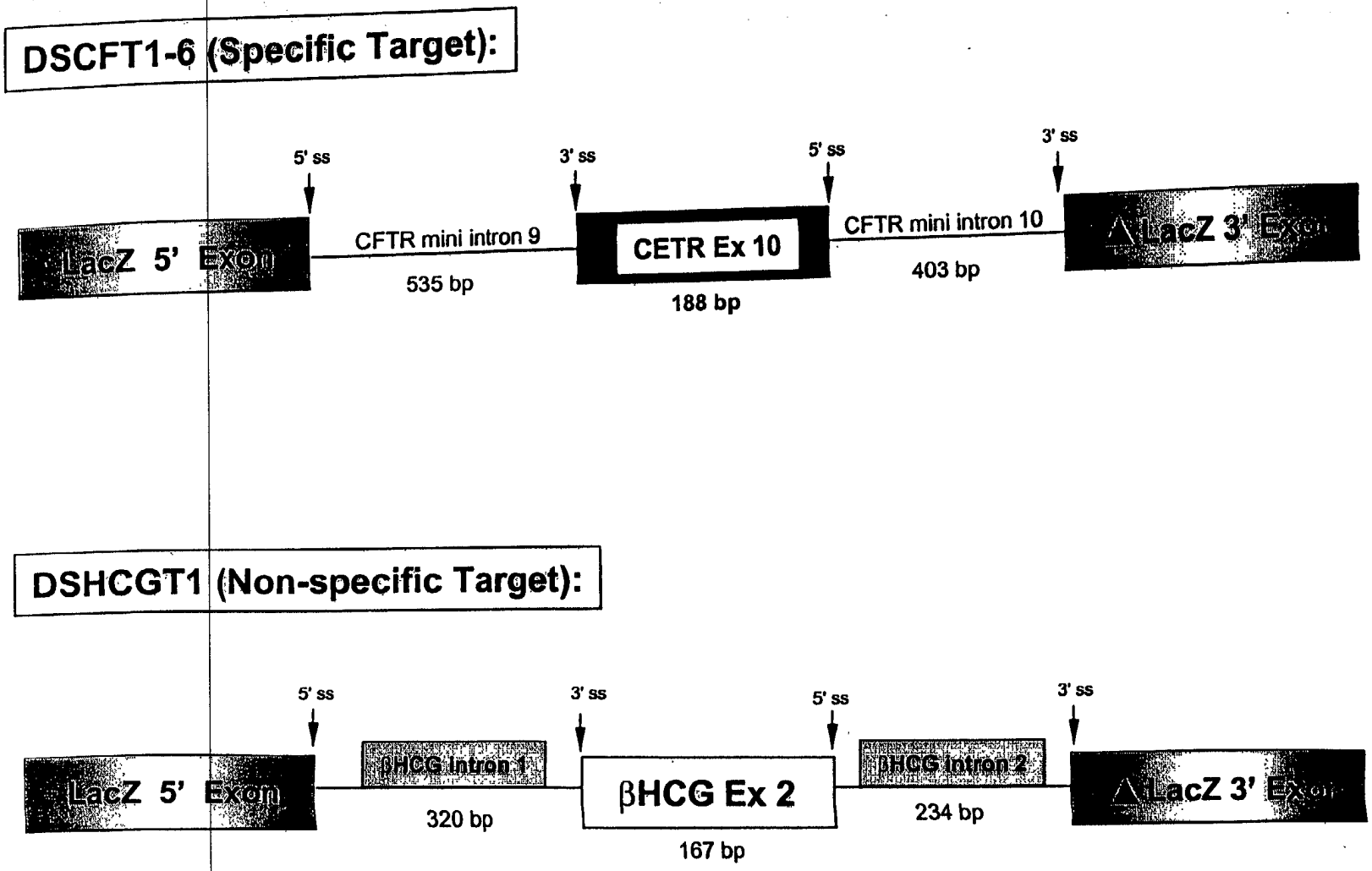

DSCFT1-6 (Specific Target):
5' ss
3' ss
5' ss
3' ss
LacZ 5' Exon
CFTR mini intron 9
535 bp
CETR Ex 10
188 bp
CFTR mini intron 10
403 bp
ΔLacZ 3' Exon
DSHCGT1 (Non-specific Target):
5' ss
3' ss
5' ss
3' ss
LacZ 5' Exon
βHCG intron 1
320 bp
βHCG Ex 2
167 bp
βHCG intron 2
234 bp
ΔLacZ 3' Exon

Figure 28

Specificity of double *trans*-splicing Reaction

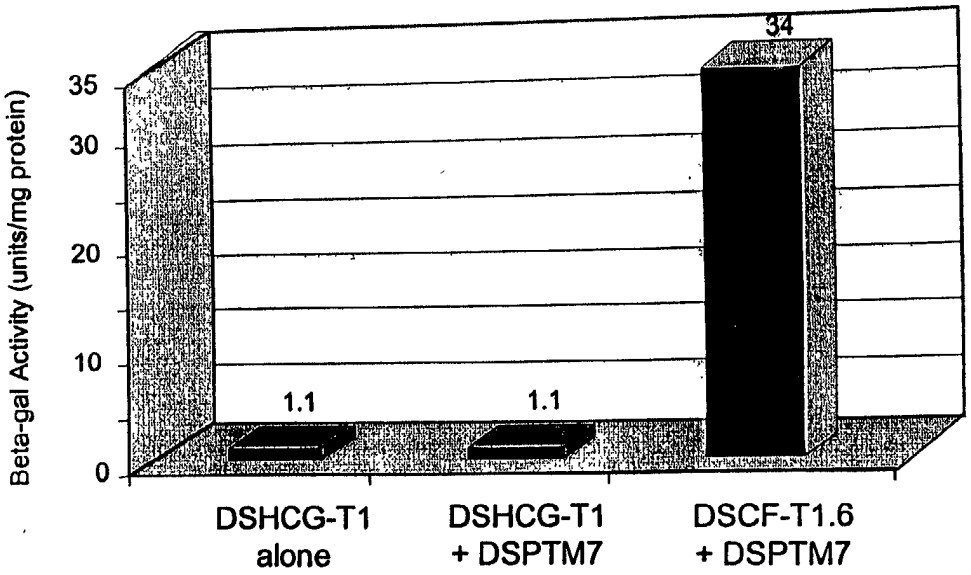
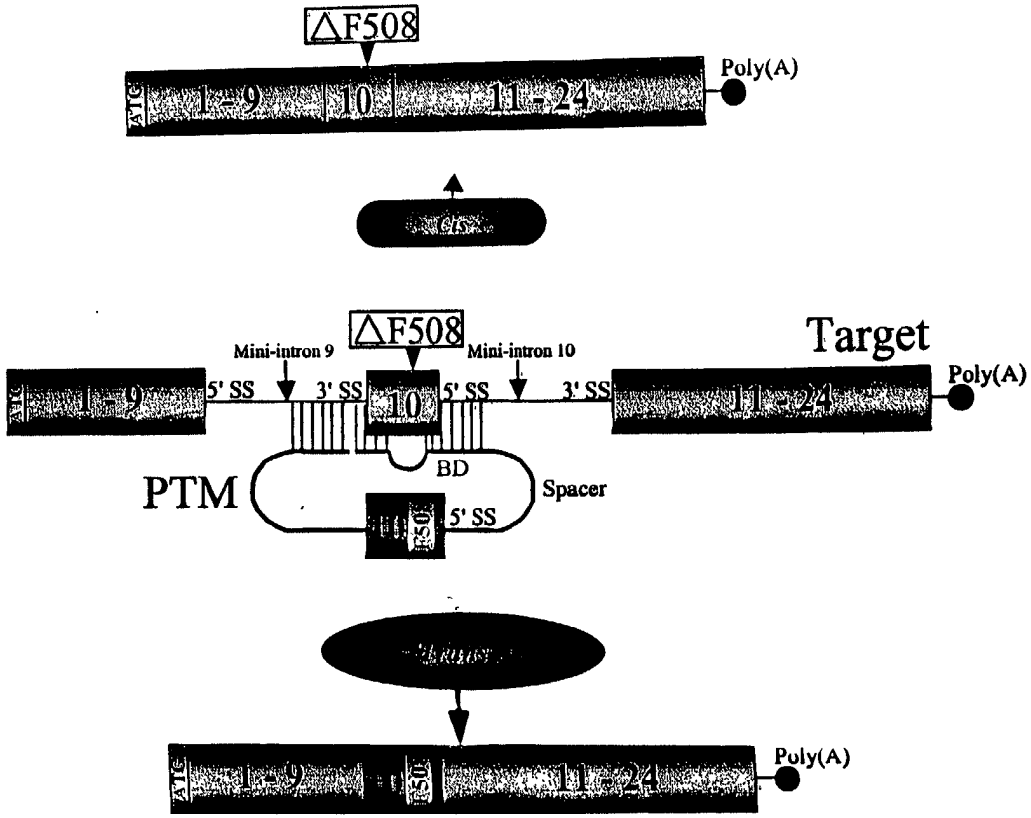


Figure 29

Replacement of a Single Internal Exon

Schematic diagram of a PTM binding to a CFTR AT-AC target

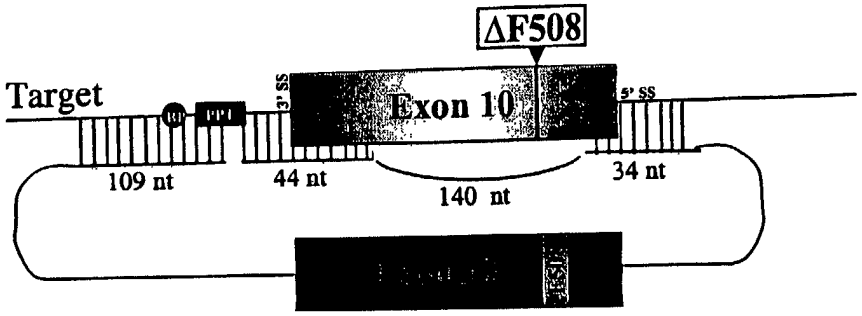


Repaired full length CFTR mRNA

Figure 30

INTRONN

PTM with a long binding domain masking two splice sites and part of exon 10 in a mini-gene target.



PTM

ACGAGCTTGCTCATGATGATCATGGGCGAGTTAGAACCAAGTGAAGGCAAGATCAAACATTCCG
GCCGCATCAGCTTTTGCAGCCAATTCAGTTGGATCATGCCCGGTACCATCAAGGAGAACATAAT
CTTCGGCGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCTGTCAGTTGGAGGAG

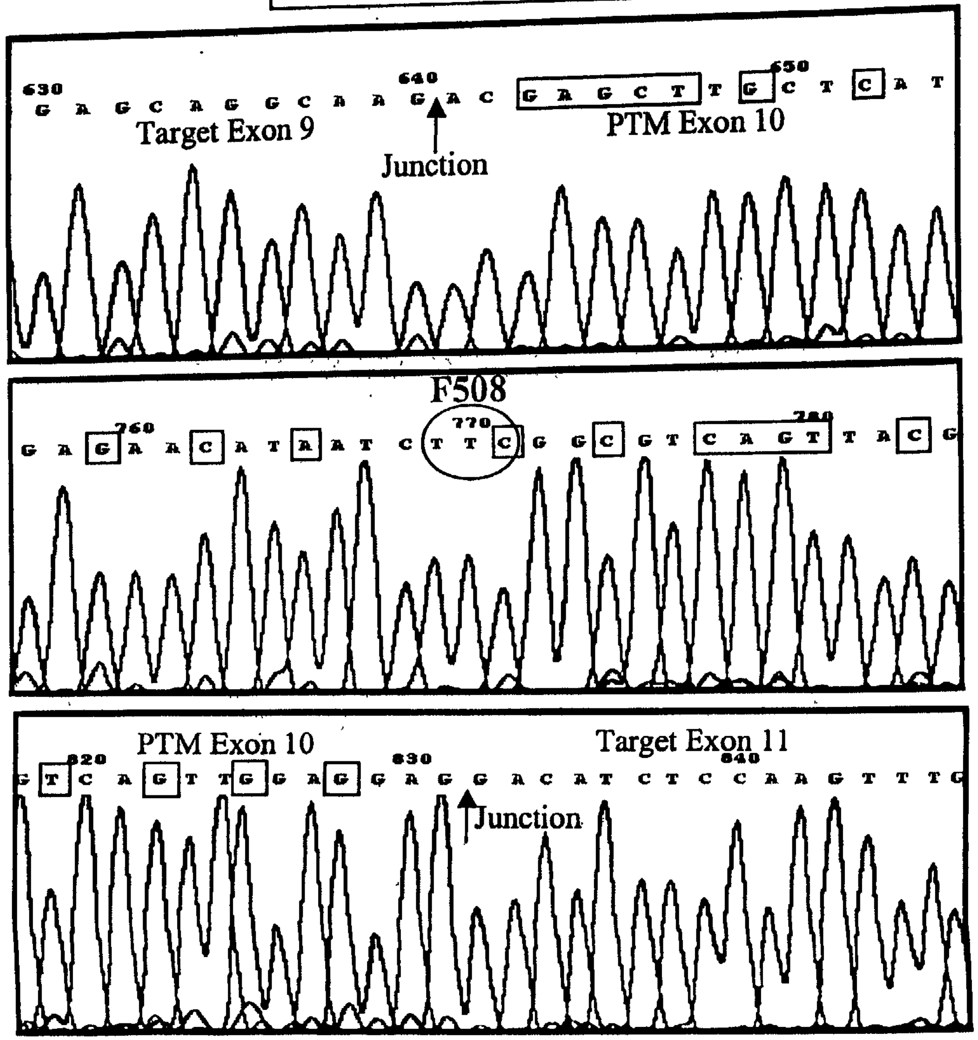
MCU in exon 10 of PTM

88 of 192 (46%) bases in PTM exon 10 are not complementary to its binding domain (bold and underlined).

Figure 31

INTRONN

Sequence of a double *trans*-spliced product

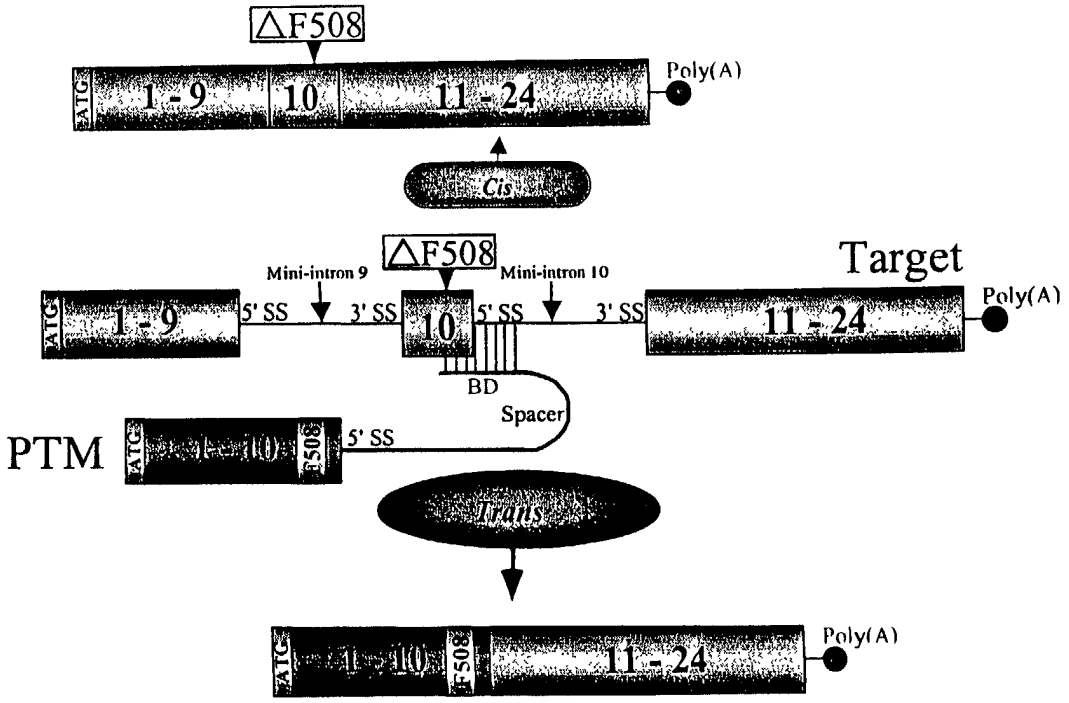


□ = MCU in PTM exon 10

Figure 32

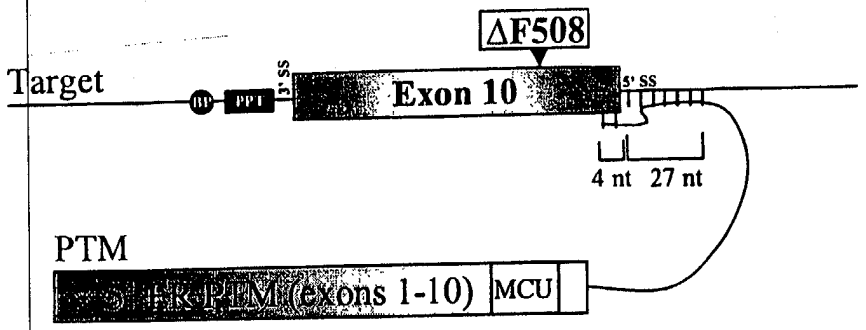
CFTR Repair: 5' Exon Replacement

Schematic diagram of a PTM binding to the splice site of intron 10 of a mini-gene target



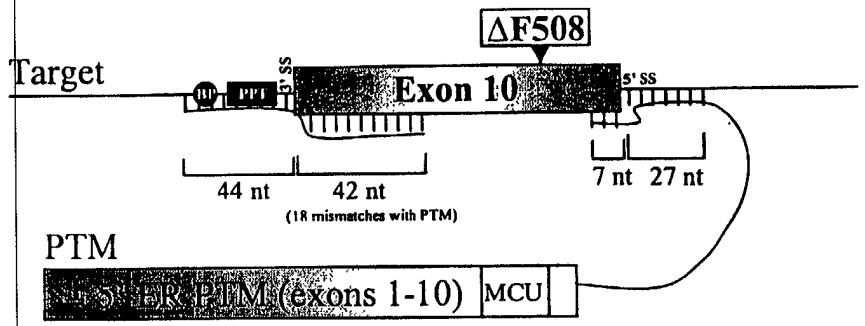
Repaired full length CFTR mRNA

Figure 33



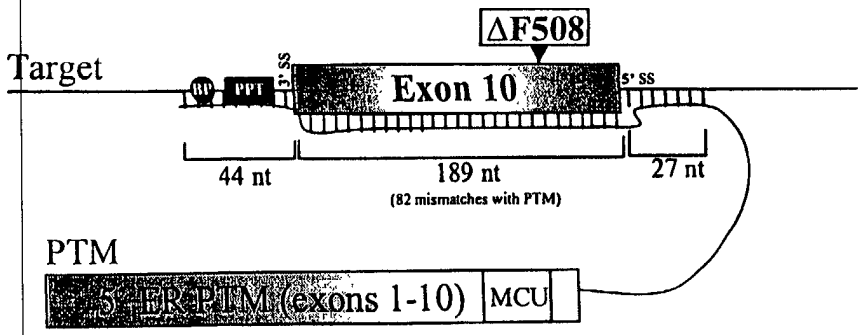
A

PTM with a short binding domain masking a single splice site in a mini-gene target.



B

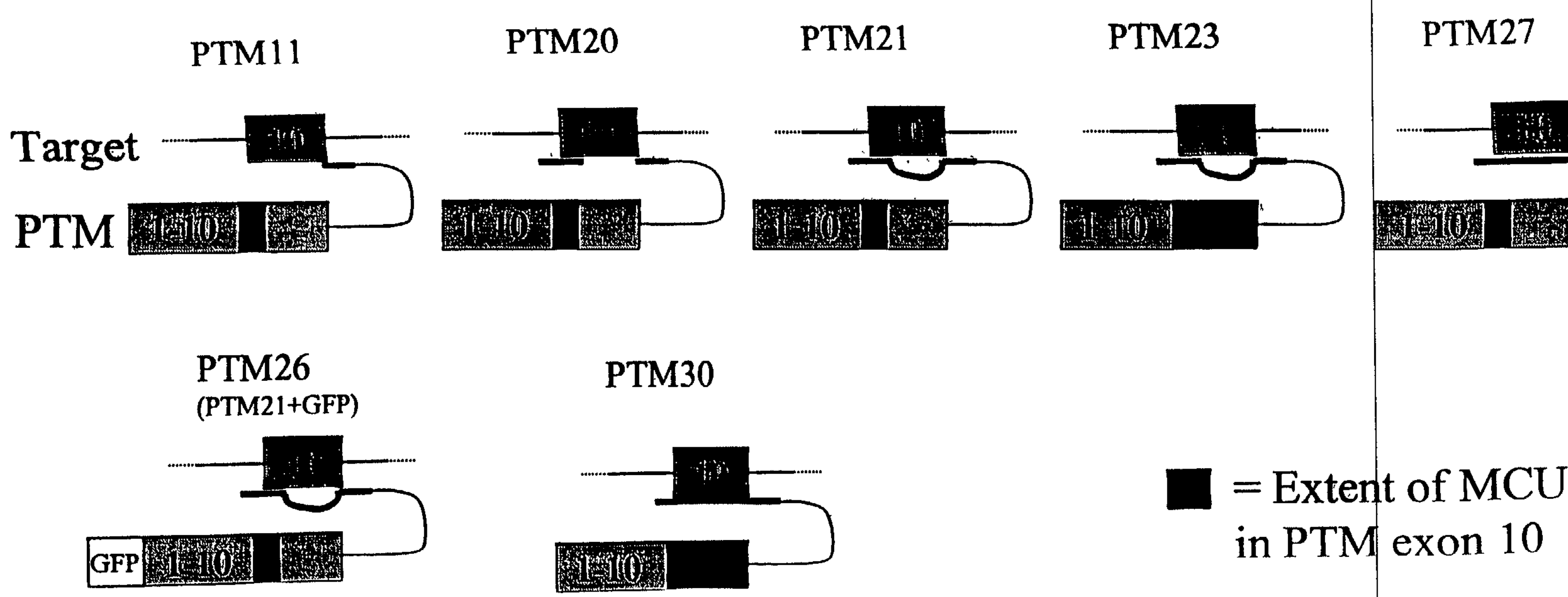
PTM with a long binding domain masking two splice sites in a mini-gene target.



C

PTM with a long binding domain masking two splice sites and the whole of exon 10 in a mini-gene target.

Figure 34



<u>MCU in exon 10 of PTM</u>

88 of 192 (46%) bases in PTM exon 10 are not complementary to its binding domain.

ACGAGCTTGCTCATGATGATCATGGGCGAGTTAGAACCAAGTGAAGGCAAGATCAAACATTCCG
GCCGCATCAGCTTTTGCAGCCAATTCAGTTGGATCATGCCCGGTACCATCAAGGAGAACATAAT
CTTCGGCGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCTGTCAGTTGGAGGAG

Figure 35

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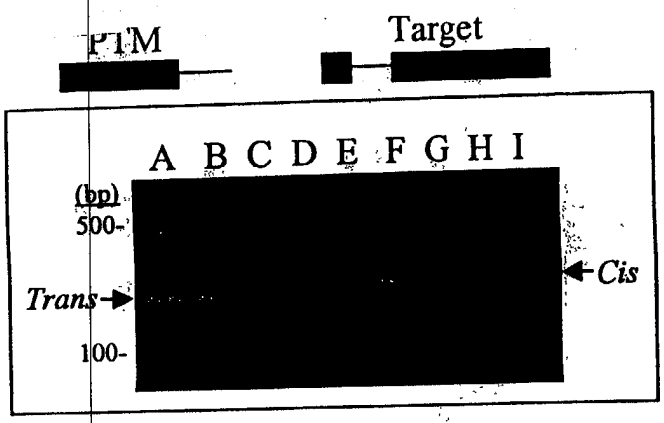
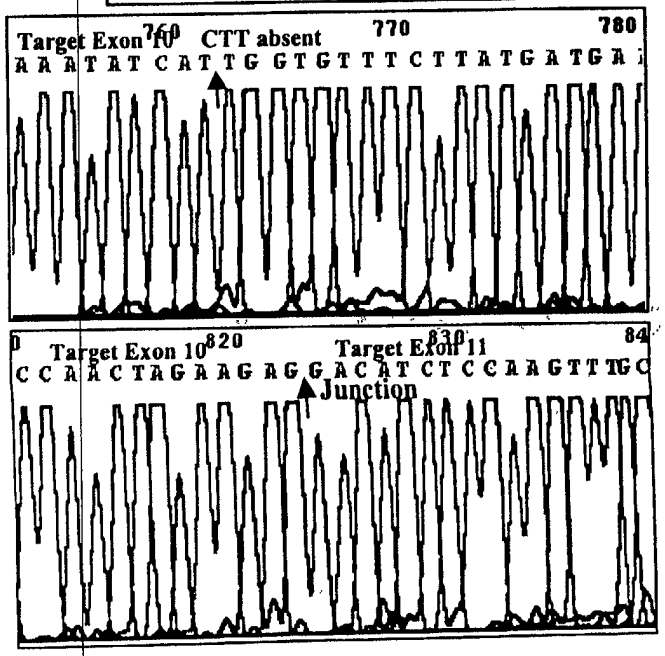
09839958 .042001



A. *Cis*-spliced product
[Primers CF1 + CF111]



B. *Trans*-spliced product
[Primers CF93 + CF111]

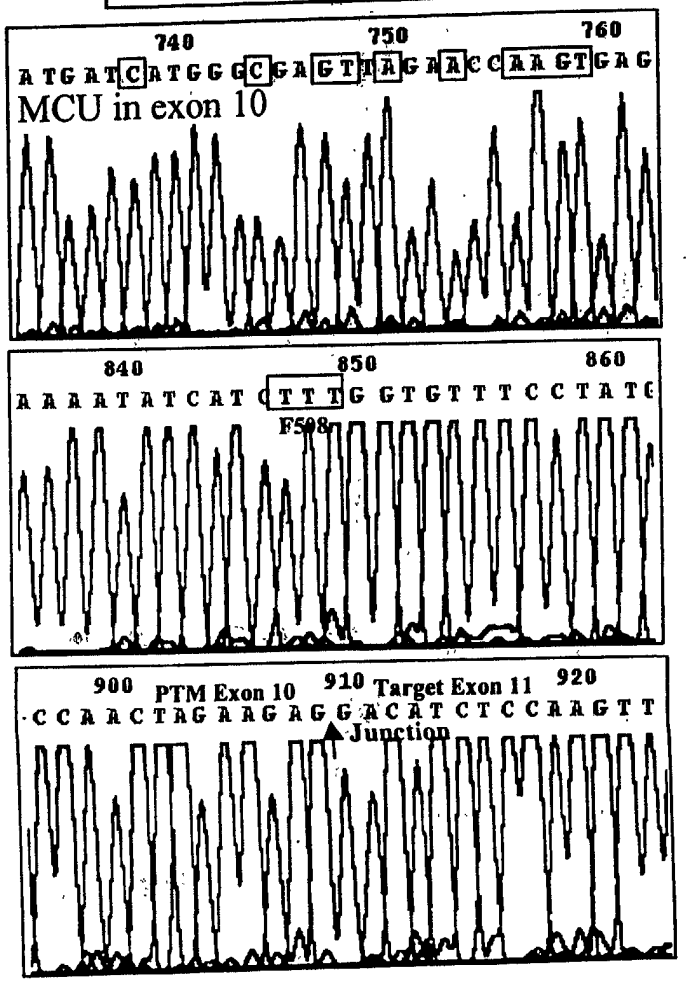


Figure 36

A

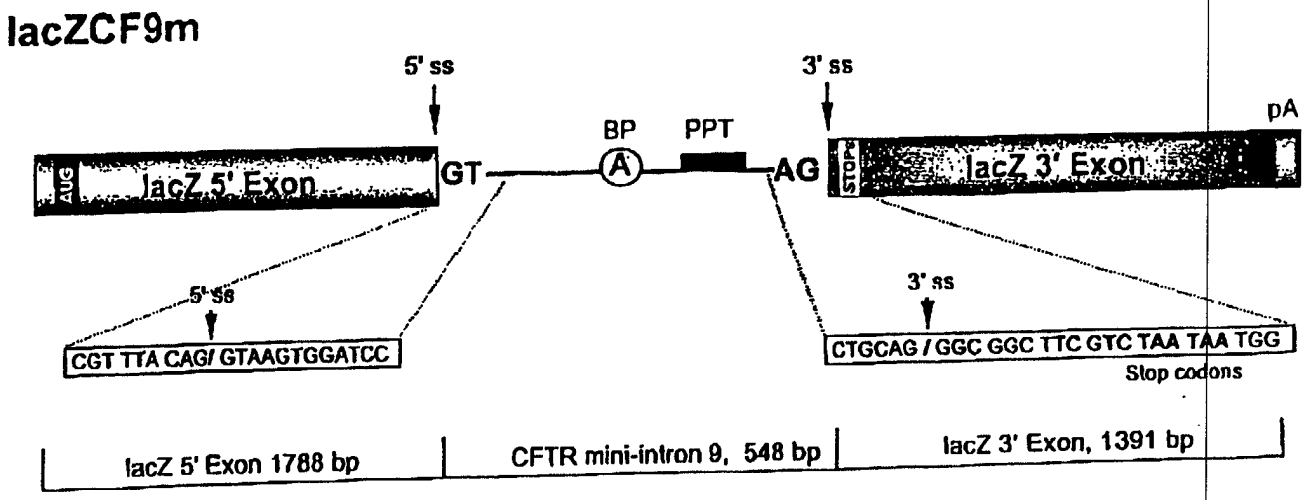
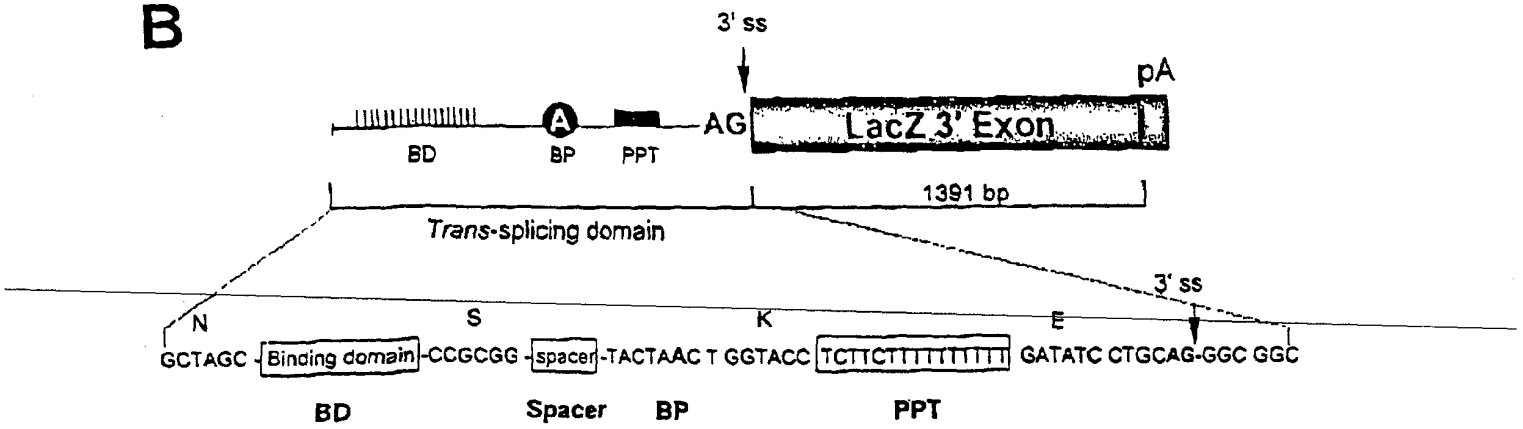

lacZCF9m
5' ss
3' ss
BP
PPT
pA
AUG
lacZ 5' Exon
GT
A
AG
STOP
lacZ 3' Exon
5' ss
3' ss
CGT TTA CAG/ GTAAGTGGATCC
CTGCAG / GGC GGC TTC GTC TAA TAA TGG
Stop codons
lacZ 5' Exon 1788 bp
CFTR mini-intron 9, 548 bp
lacZ 3' Exon, 1391 bp

Figure 37 A


B
3' ss
pA
AG
LacZ 3' Exon
BD
BP
PPT
1391 bp
Trans-splicing domain
3' ss
N
S
K
E
GCTAGC -Binding domain-CCGCGG -spacer-TACTAAC T GGTACC TCTTCTTTTTTTTTT GATATC CTGCAG-GGC GGC
BD
Spacer
BP
PPT

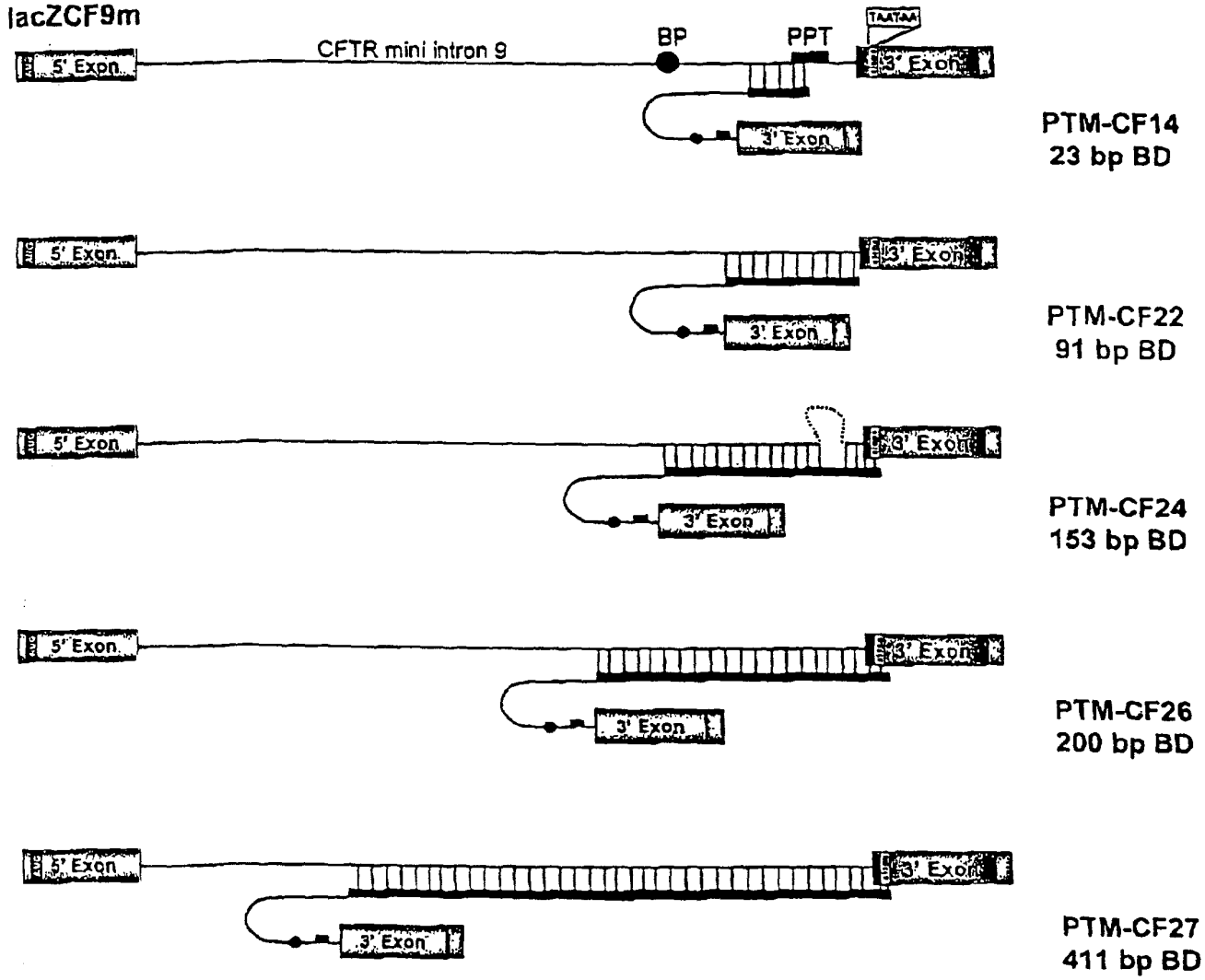

lacZCF9m
5' Exon
CFTR mini intron 9
BP
PPT
TAATAA
3' Exon
3' Exon
PTM-CF14
23 bp BD
5' Exon
3' Exon
3' Exon
PTM-CF22
91 bp BD
5' Exon
3' Exon
3' Exon
PTM-CF24
153 bp BD
5' Exon
3' Exon
3' Exon
PTM-CF26
200 bp BD
5' Exon
3' Exon
3' Exon
PTM-CF27
411 bp BD

Figure 37B

C

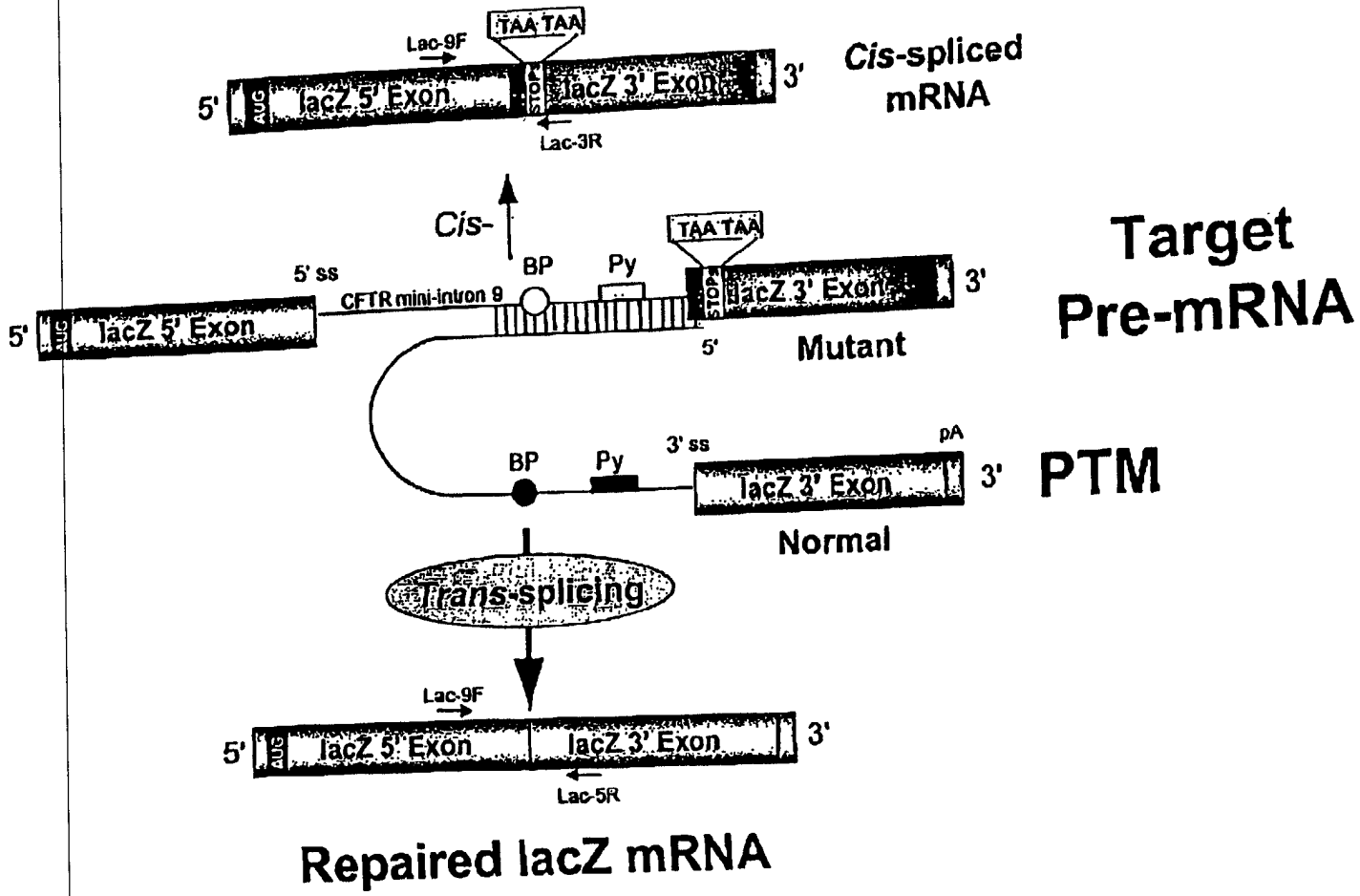
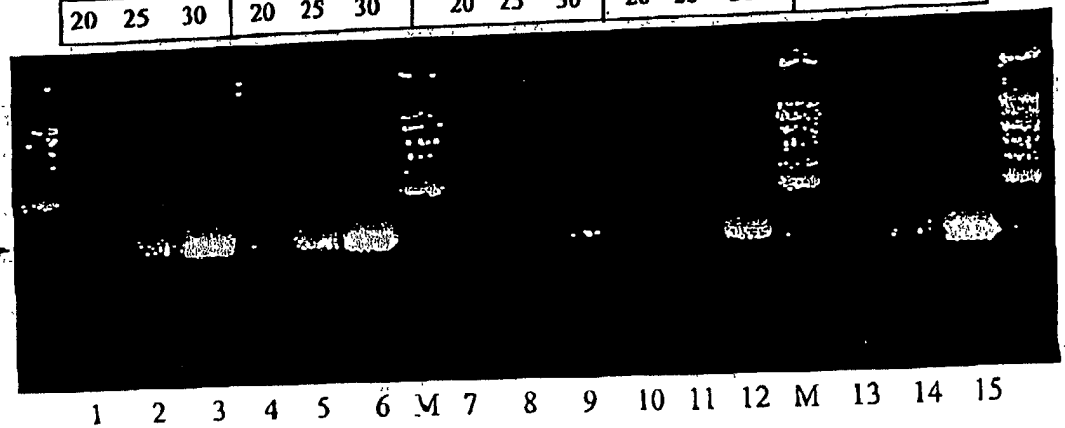


Figure 37C

A

Cis-splicing	*Trans*-splicing			
lacZCF9r: + PTM-CF14				lacZCF9
25 ng	50 ng	100 ng	200 ng	25 ng
20 25 30	20 25 30	20 25 30	20 25 30	20 25 30

Total RNA
PCR cycles



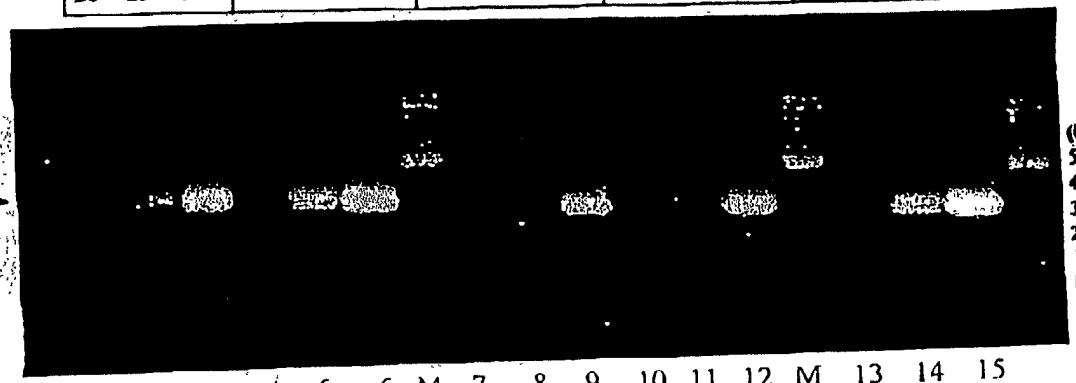
Cis-spliced
(303 bp)

Trans-spliced
(299 bp)

Figure 38 A

Cis-splicing	*Trans*-splicing			
lacZCF9m + PTM-CF24				lacZCF9
25 ng	50 ng	50 ng	100 ng	50 ng
20 25 30	20 25 30	20 25 30	20 25 30	20 25 30

Total RNA
PCR cycles



Cis-spliced
(303 bp)

Trans-spliced
(299 bp)

Shut 47 of 66

B

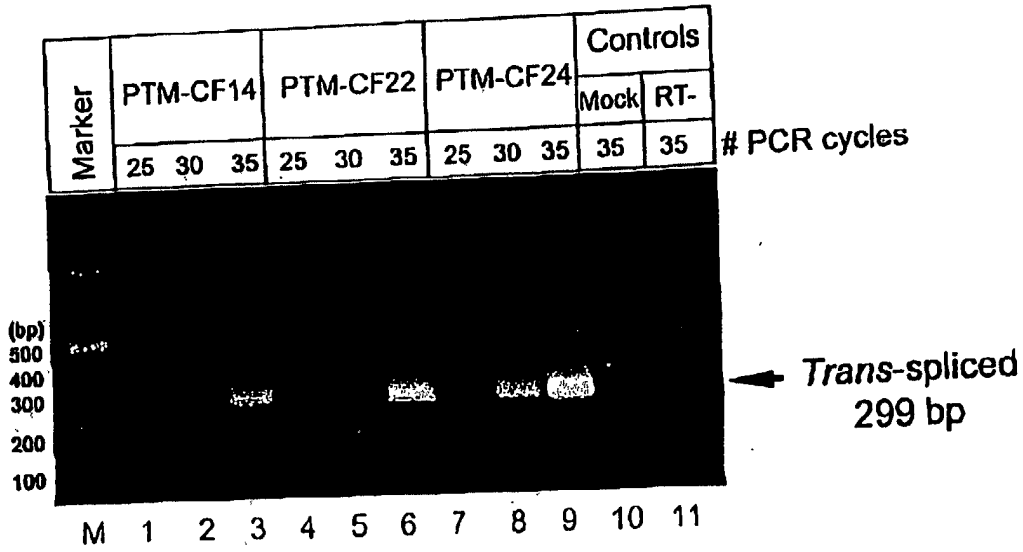

Marker
PTM-CF14
PTM-CF22
PTM-CF24
Controls
Mock
RT-
25 30 35 25 30 35 25 30 35 35 35
PCR cycles
(bp)
500
400
300
200
100
Trans-spliced
299 bp
M 1 2 3 4 5 6 7 8 9 10 11

Figure 38B

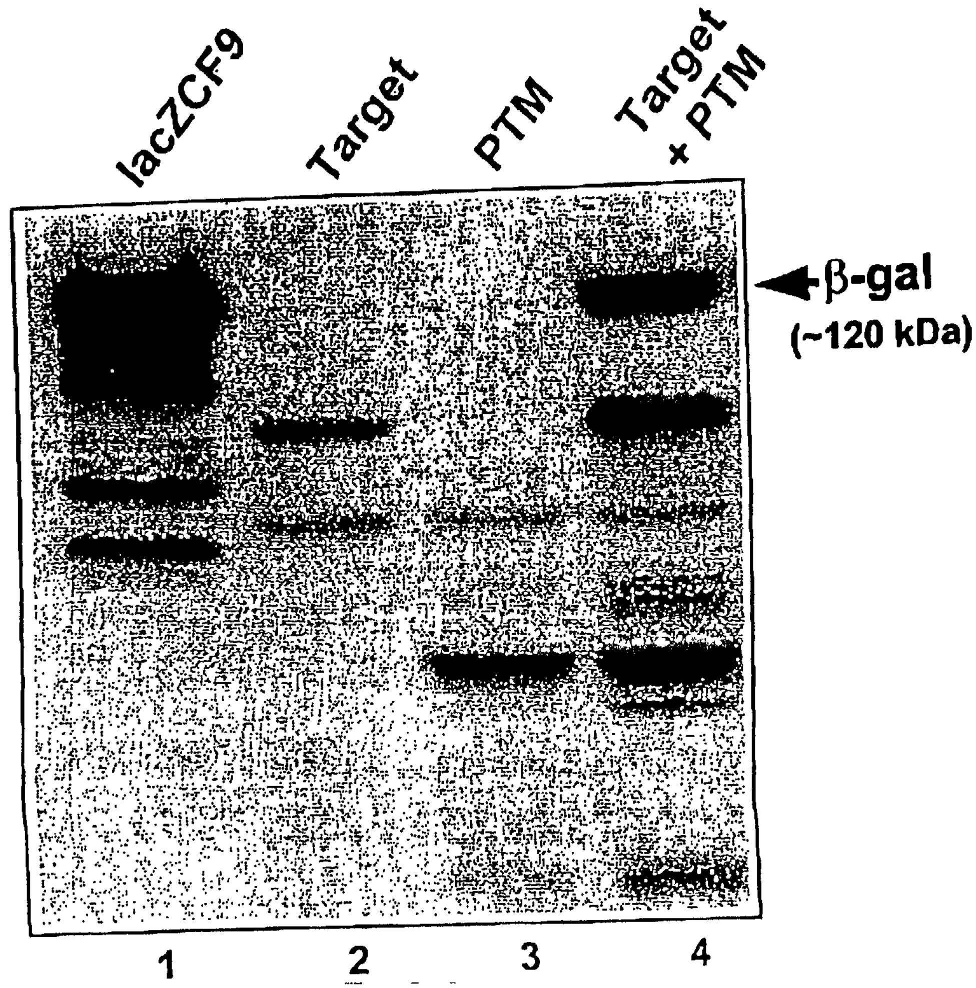


Figure 39

Figure 40A

B
β-gal activity (units/mg protein)
2500
2000
1500
1000
500
0
Target
PTM
Controls
14
22
24
26
27
lacZCF9m + Linear PTM

Figure 40B

C

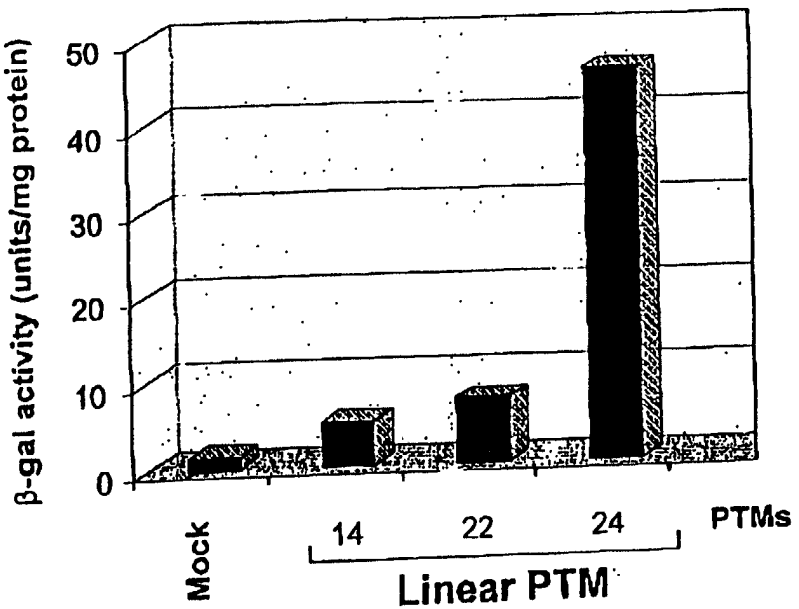


Figure 40C

Shut 52 of 99

A

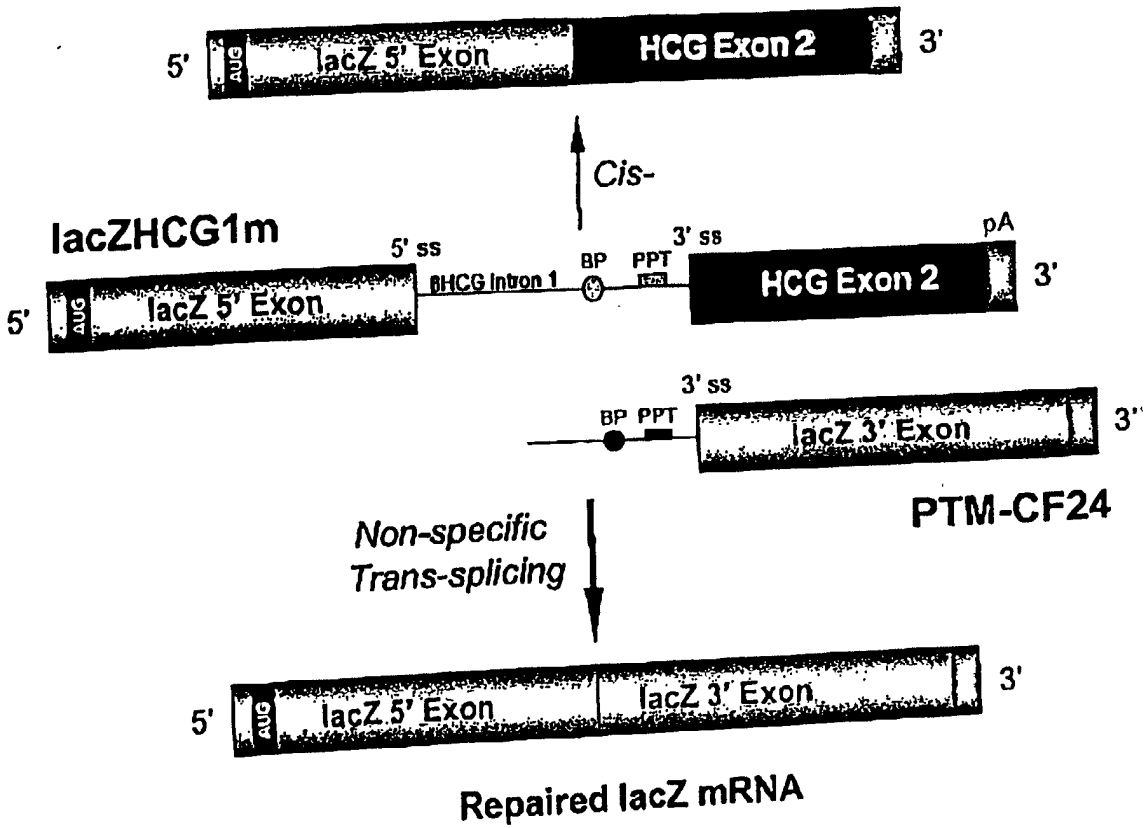


Figure 41A

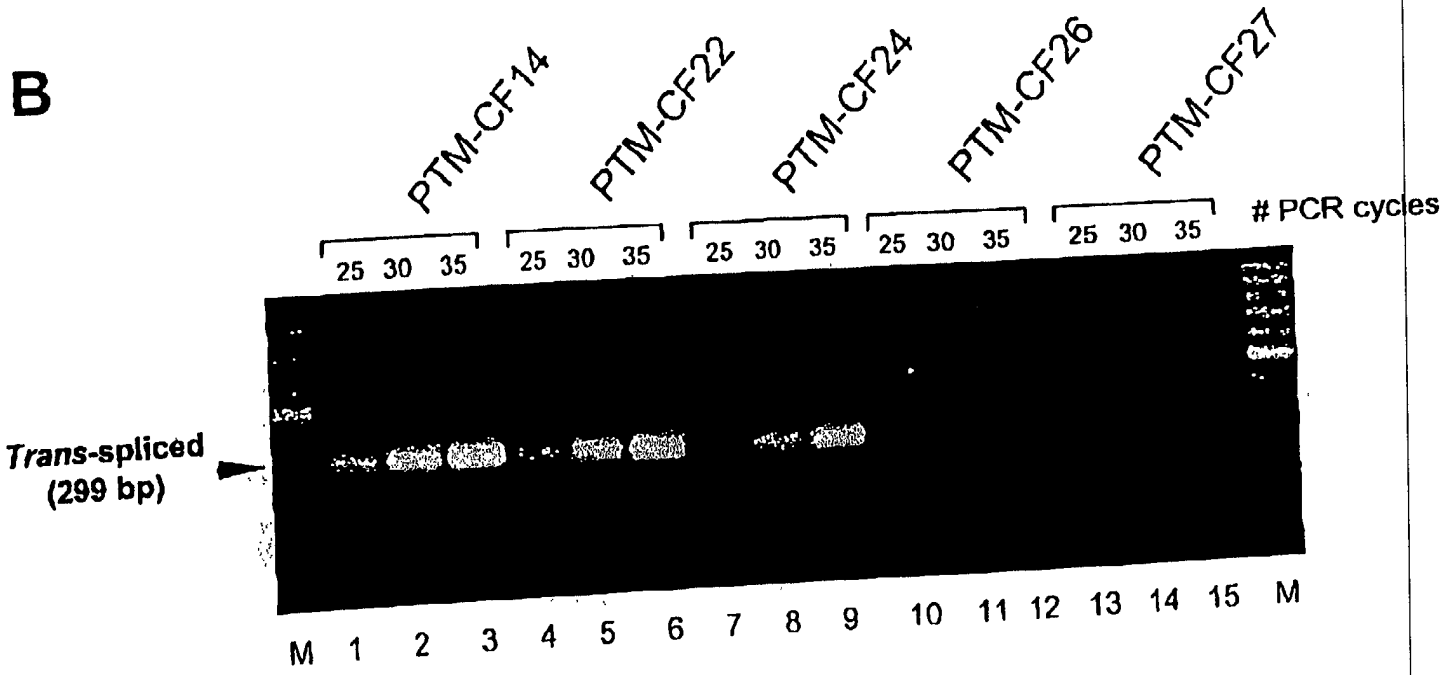


Figure 4B

Shut 54 of 96

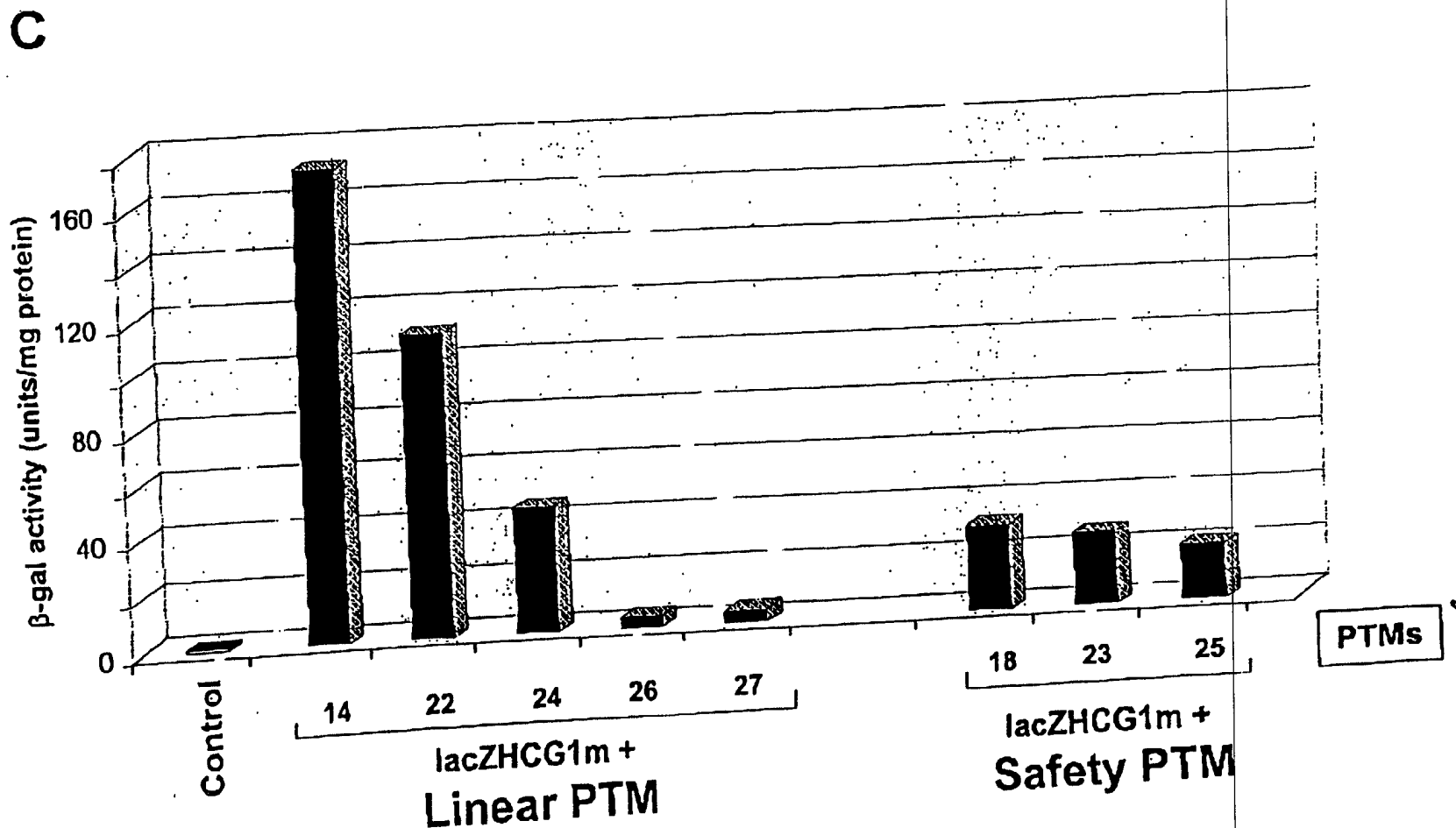


Figure 41C

Exons 1-10
ATGCAGAGGTCGCCTCTGGAAAAGGCCAGCGTTGTCTCCAAACTTTTTTTCAGCTGGACCAGACCAATTTTGAGGAAAG
GATACAGACAGCGCCTGGAATTGTCAGACATATACCAAATCCCTTCTGTTGATTCTGCTGACAATCTATCTGAAAAATT
GGAAAGAGAATGGGATAGAGAGCTGGCTTCAAAGAAAAATCCTAAACTCATTAATGCCCTTCGGCGATGTTTTTTCTGG
AGATTTATGTTCTATGGAATCTTTTTATATTTAGGGGAAGTCACCAAAGCAGTACAGCCTCTCTTACTGGAAGAATCA
TAGCTTCCTATGACCCGGATAACAAGGAGGAACGCTCTATCGCGATTTATCTAGGCATAGGCTTATGCCTTCTCTTTAT
TGTGAGGACACTGCTCCTACACCCAGCCATTTTTGGCCTTCATCACATTGGAATGCAGATGAGAATAGCTATGTTTAGT
TTGATTTATAAGAAGACTTTAAAGCTGTCAAGCCGTGTTCTAGATAAAATAAGTATTGGACAACTTGTTAGTCTCCTTT
CCAACAACCTGAACAAATTTGATGAAGGACTTGCATTGGCACATTTCGTGTGGATCGCTCCTTTGCAAGTGGCACTCCT
CATGGGGCTAATCTGGGAGTTGTTACAGGCGTCTGCCTTCTGTGGACTTGGTTTCCTGATAGTCCTTGCCCTTTTTCAG
GCTGGGCTAGGGAGAATGATGATGAAGTACAGAGATCAGAGAGCTGGGAAGATCAGTGAAAGACTTGTGATTACCTCAG
AAATGATCGAGAACATCCAATCTGTTAAGGCATACTGCTGGGAAGAAGCAATGGAAAAAATGATTGAAAACTTAAGACA
AACAGAACTGAAACTGACTCGGAAGGCAGCCTATGTGAGATACTTCAATAGCTCAGCCTTCTTCTTCTCAGGGTTCTTT
GTGGTGTTTTTATCTGTGCTTCCCTATGCACTAATCAAAGGAATCATCCTCCGGAAAATATTCACCACCATCTCATTCT
GCATTGTTCTGCGCATGGCGGTCACTCGGCAATTTCCCTGGGCTGTACAAACATGGTATGACTCTCTTGGAGCAATAAA
CAAAATACAGGATTTCTTACAAAAGCAAGAATATAAGACATTGGAATATAACTTAACGACTACAGAAGTAGTGATGGAG
AATGTAACAGCCTTCTGGGAGGAGGGATTTGGGGAATTATTTGAGAAAGCAAAACAAAACAATAACAATAGAAAAACTT
CTAATGGTGATGACAGCCTCTTCTTCAGTAATTTCTCACTTCTTGGTACTCCTGTCCTGAAAGATATTAATTTCAAGAT
AGAAAGAGGACAGTTGTTGGCGGTTGCTGGATCCACTGGAGCAGGCAAGACGAGCTTGCTCATGATGATCATGGGCGAG
TTAGAACCAAGTGAAGGCAAGATCAAACATTCCGGCCGCATCAGCTTTTGCAGCCAATTCAGTTGGATCATGCCCGGTA
CCATCAAGGAGAACATAATCTTCGGCGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCTGTCAGTTGGA
GGAG
Trans-splicing domain
GTAAGATATCACCGATATGTGTCTAACCTGATTCGGGCCTTCGATACGCTAAGATCCACCGG
TCAAAAAGTTTTCACATAATTTCTTACCTCTTCTTGAATTCATGCTTTGATGACGCTTCTGTATCTATATTCATCATTG
GAAACACCAATGATATTTTCTTTAATGGTGCCTGGCATAATCCTGGAAAACTGATAACACAATGAAATTCTTCCACTGT
GCTTAATTTTACCCTCTGAATTCTCCATTTCTCCCATAATCATCATTACAACTGAACTCTGGAAATAAAACCCATCATT
ATTAACTCATTATCAAATCACGCT

Figure 42

153 bp PTM24 Binding Domain:

Nhe I 153 bp BD underlined

GCTAGC-AATAATGACGAAGCCGCCCCTCACGCTCAGGATTCACTTGCCTCCAATTATCATCCTAAGCAGAAGTGTATA

TTCTTATTTGTAAAGATTCTATTAACTCATTTGATTCAAAATATTTAAAATACTTCCTGTTTCACCTACTCTGCTATGC

Sac II
AC-**CCGCGG**

Figure 43A

Trans-splicing domain
AATAATGACGAAGCCGCCCCTCACGCTCAGGATTCACTTGCCCTCCAATTATCATCCTAAGCAGAAGTGTATATTCTTA
TTTGTAAAGATTCTATTAACTCATTTGATTCAAAATATTTAAAATACTTCCTGTTTCACCTACTCTGCTATGCACCCGC
GGAACATTATTATAACGTTGCTCGAATACTAACTGGTACCTCTTCTTTTTTTTTGATATCCTGCAG
Exons 10-24
ACTTCACTTCTAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAATTAAGCACAGTGGAAGAATTTCATTCT
GTTCTCAGTTTTCCTGGATTATGCCTGGCACCATTAAAGAAAATATCATCTTTGGTGTTTCCTATGATGAATATAGATA
CAGAAGCGTCATCAAAGCATGCCAACTAGAAGAGGACATCTCCAAGTTTGCAGAGAAAGACAATATAGTTCTTGGAGAA
GGTGGAATCACACTGAGTGGAGGTCAACGAGCAAGAATTTCTTTAGCAAGAGCAGTATACAAAGATGCTGATTTGTATT
TATTAGACTCTCCTTTTGGATACCTAGATGTTTTAACAGAAAAAGAAATATTTGAAAGCTGTGTCTGTAAACTGATGGC
TAACAAAACTAGGATTTTGGTCACTTCTAAAATGGAACATTTAAAGAAAGCTGACAAAATATTAATTTTGCATGAAGGT
AGCAGCTATTTTTATGGGACATTTTCAGAACTCCAAAATCTACAGCCAGACTTTAGCTCAAAACTCATGGGATGTGATT
CTTTCGACCAATTTAGTGCAGAAAGAAGAAATTCAATCCTAACTGAGACCTTACACCGTTTCTCATTAGAAGGAGATGC
TCCTGTCTCCTGGACAGAAACAAAAAAACAATCTTTTAAACAGACTGGAGAGTTTGGGGAAAAAAGGAAGAATTCTATT
CTCAATCCAATCAACTCTATACGAAAATTTTCCATTGTGCAAAAGACTCCCTTACAAATGAATGGCATCGAAGAGGATT
CTGATGAGCCTTTAGAGAGAAGGCTGTCCTTAGTACCAGATTCTGAGCAGGGAGAGGCGATACTGCCTCGCATCAGCGT
GATCAGCACTGGCCCCACGCTTCAGGCACGAAGGAGGCAGTCTGTCCTGAACCTGATGACACACTCAGTTAACCAAGGT
CAGAACATTCACCGAAAGACAACAGCATCCACACGAAAAGTGTCACTGGCCCCTCAGGCAAACTTGACTGAACTGGATA
TATATTCAAGAAGGTTATCTCAAGAAACTGGCTTGGAAATAAGTGAAGAAATTAACGAAGAAGACTTAAAGGAGTGCTT
TTTTGATGATATGGAGAGCATACCAGCAGTGACTACATGGAACACATACCTTCGATATATTACTGTCCACAAGAGCTTA
ATTTTTGTGCTAATTTGGTGCTTAGTAATTTTTCTGGCAGAGGTGGCTGCTTCTTTGGTTGTGCTGTGGCTCCTTGGAA
ACACTCCTCTTCAAGACAAAGGGAATAGTACTCATAGTAGAAATAACAGCTATGCAGTGATTATCACCAGCACCAGTTC
GTATTATGTGTTTTACATTTACGTGGGAGTAGCCGACACTTTGCTTGCTATGGGATTCTTCAGAGGTCTACCACTGGTG
CATACTCTAATCACAGTGTCGAAAATTTTACACCACAAAATGTTACATTCTGTTCTTCAAGCACCTATGTCAACCCTCA
ACACGTTGAAAGCAGGTGGGATTCTTAATAGATTCTCCAAAGATATAGCAATTTTGGATGACCTTCTGCCTCTTACCAT
ATTTGACTTCATCCAGTTGTTATTAATTGTGATTGGAGCTATAGCAGTTGTCGCAGTTTTACAACCCTACATCTTTGTT
GCAACAGTGCCAGTGATAGTGGCTTTTATTATGTTGAGAGCATATTTCCTCCAAACCTCACAGCAACTCAAACAACTGG
AATCTGAAGGCAGGAGTCCAATTTTCACTCATCTTGTTACAAGCTTAAAAGGACTATGGACACTTCGTGCCTTCGGACG
GCAGCCTTACTTTGAAACTCTGTTCCACAAAGCTCTGAATTTACATACTGCCAACTGGTTCTTGTACCTGTCAACACTG
CGCTGGTTCCAAATGAGAATAGAAATGATTTTTGTCATCTTCTTCATTGCTGTTACCTTCATTTCCATTTTAACAACAG
GAGAAGGAGAAGGAAGAGTTGGTATTATCCTGACTTTAGCCATGAATATCATGAGTACATTGCAGTGGGCTGTAAACTC
CAGCATAGATGTGGATAGCTTGATGCGATCTGTGAGCCGAGTCTTTAAGTTCATTGACATGCCAACAGAAGGTAAACCT
ACCAAGTCAACCAAACCATACAAGAATGGCCAACTCTCGAAAGTTATGATTATTGAGAATTCACACGTGAAGAAAGATG
ACATCTGGCCCTCAGGGGGCCAAATGACTGTCAAAGATCTCACAGCAAAATACACAGAAGGTGGAAATGCCATATTAGA
GAACATTTCCTTCTCAATAAGTCCTGGCCAGAGGGTGGGCCTCTTGGGAAGAACTGGATCAGGGAAGAGTACTTTGTTA
TCAGCTTTTTTGAGACTACTGAACACTGAAGGAGAAATCCAGATCGATGGTGTGTCTTGGGATTCAATAACTTTGCAAC
AGTGGAGGAAAGCCTTTGGAGTGATACCACAGAAAGTATTTATTTTTTCTGGAACATTTAGAAAAAACTTGGATCCCTA
TGAACAGTGGAGTGATCAAGAAATATGGAAAGTTGCAGATGAGGTTGGGCTCAGATCTGTGATAGAACAGTTTCCTGGG
AAGCTTGACTTTGTCCTTGTGGATGGGGGCTGTGTCCTAAGCCATGGCCACAAGCAGTTGATGTGCTTGGCTAGATCTG
TTCTCAGTAAGGCGAAGATCTTGCTGCTTGATGAACCCAGTGCTCATTTGGATCCAGTAACATACCAAATAATTAGAAG
AACTCTAAAACAAGCATTTGCTGATTGCACAGTAATTCTCTGTGAACACAGGATAGAAGCAATGCTGGAATGCCAACAA
TTTTTGGTCATAGAAGAGAACAAAGTGCGGCAGTACGATTCCATCCAGAAACTGCTGAACGAGAGGAGCCTCTTCCGGC
AAGCCATCAGCCCCTCCGACAGGGTGAAGCTCTTTCCCCACCGGAACTCAAGCAAGTGCAAGTCTAAGCCCCAGATTGC
Histidine tag Stop
TGCTCTGAAAGAGGAGACAGAAGAAGAGGTGCAAGATACAAGGCTTCATCATCATCATCATCATTAG

Figure 43B

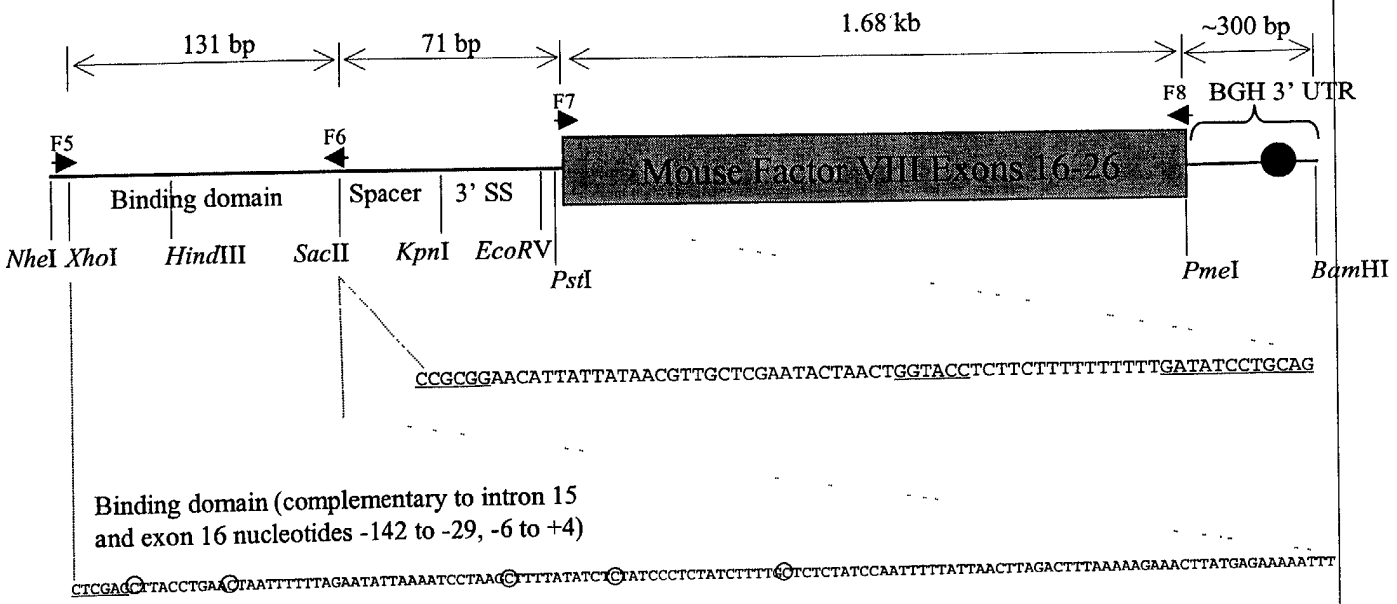


Figure 44 A

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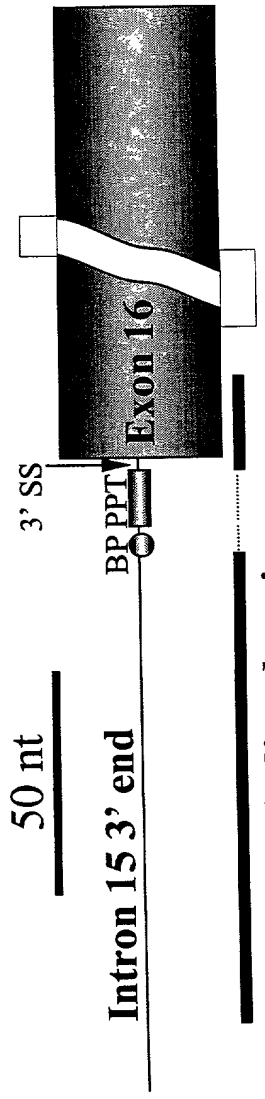


Figure 44 B

Figure 44C

Chicken β-actin Promoter

NcoI
CCATGGTCGACGTTAGCCCCACGTTCTGCTTCACTCTCCCCATCTCCCCCCCCTCCCCACCCCCAATT

TTGTATTTATTTATTTTTTAATTATTTTGTGCAGCGATGGGGGCGGGGGGGGGGGGGGGGCGCGCGCCAGGC

GGGGCGGGGCGGGGCGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGCGGCGGCAGCCAATCAGAGCGGCGCG

CTCCGAAAGTTTCCTTTTATGGCGAGGCGGCGGCGGCGGCGGCCCTATAAAAAGCGAAGCGCGCGGCGGGCG

F13 .... XhoI
GGAGTCGCTGCGACGCTGCCTTCGCCCCGTGCCAACCTCCGCCTCGAGCTTACCTGAACTAATTTTTTAGAA

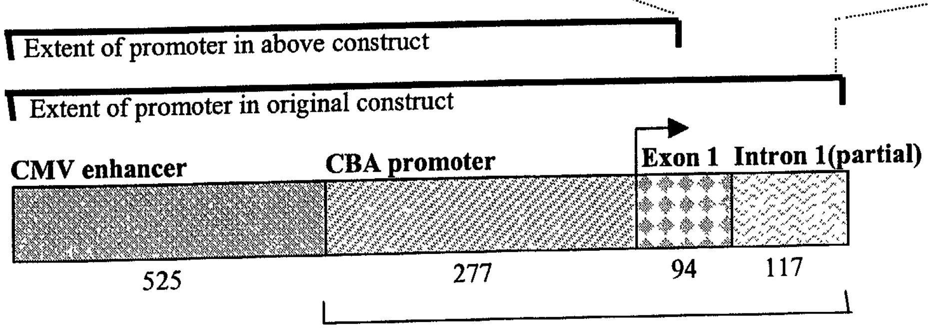
HindIII
TATTAAAATCCTAAGCTTTTATATCTCTATCCCTCTATCTTTTGCTCTCTATCCAATTTTTATTAACTTAGA

SacII
CTTTAAAAAGAAACTTATGAGAAAAATTT*CCGCGGAACATTATTATAACGTTGCTCGAATACTAACTGGTAC*

EcoRV PstI
CTCTTCTTTTTTTTTTTGATATCCTGCAG

Nucleotide changes are shown in blue
Boxed = CAT box, TATA box
Boxed + Arrow = Transcription Start
Oval = Downstream elements
Bold = Binding domain
Italicized = Spacer+PPT+BP+AG dinucleotide

Sequence not included in construct
CGCCGCCTCGCGCCGCCCGCCCCGGCTCTGACTGACCGCGTTACTCCCACAGGTGAG
CGGGCGGGACGGCCCTTCTCCTCCGGGCTGTAATTAGCGCTTGGTTTAATGACGGCT
TGTTTCTTTTCTGTGGCTGCGTGAAAGCCTTGAGGGGCTCCGGGAGGAATTCGTA



F13 + F2 = 235 + 106 = 341 bp
F13 + F4 = 235 + 315 = 550 bp

Chicken Beta Actin Promoter (including exon 1 and part of intron 1)

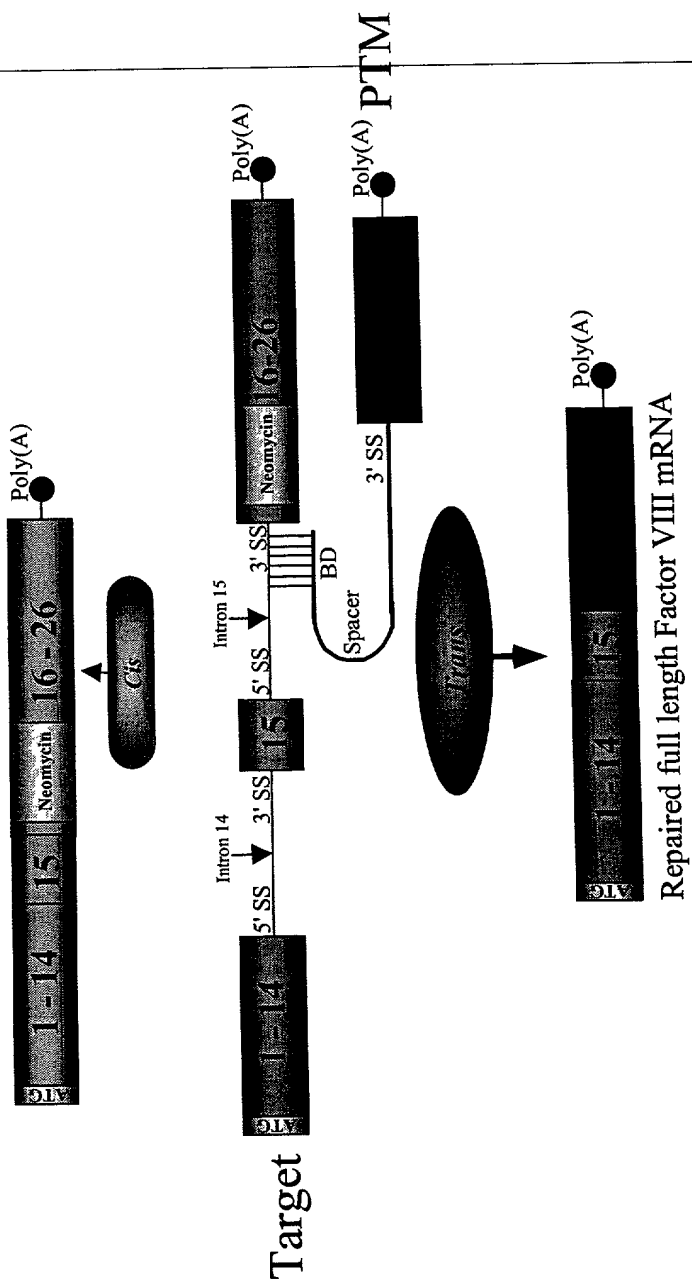
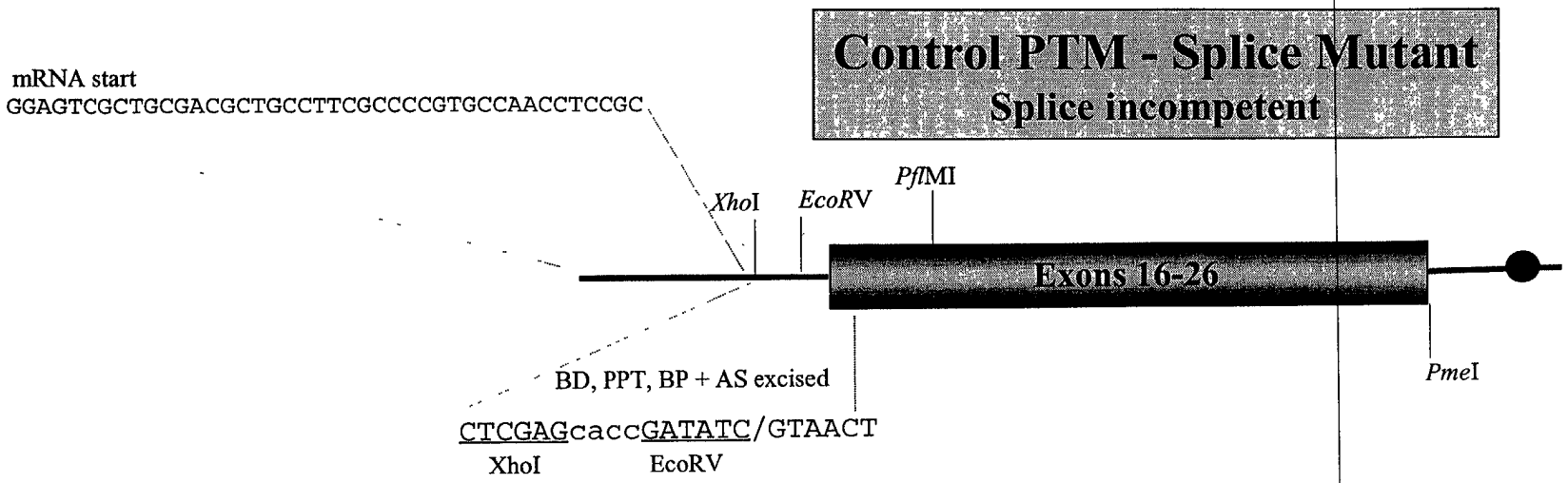

Poly(A)
1 - 14
15
Neomycin
16 - 26
ATG
Cis
Poly(A)
1 - 14
ATG
5' SS
Intron 14
3' SS
15
5' SS
Intron 15
3' SS
Neomycin
16-26
Poly(A)
BD
Spacer
3' SS
PTM
Trans
Poly(A)
1 - 14
15
ATG
Repaired full length Factor VIII mRNA
Target

Figure 44D

Figure 45

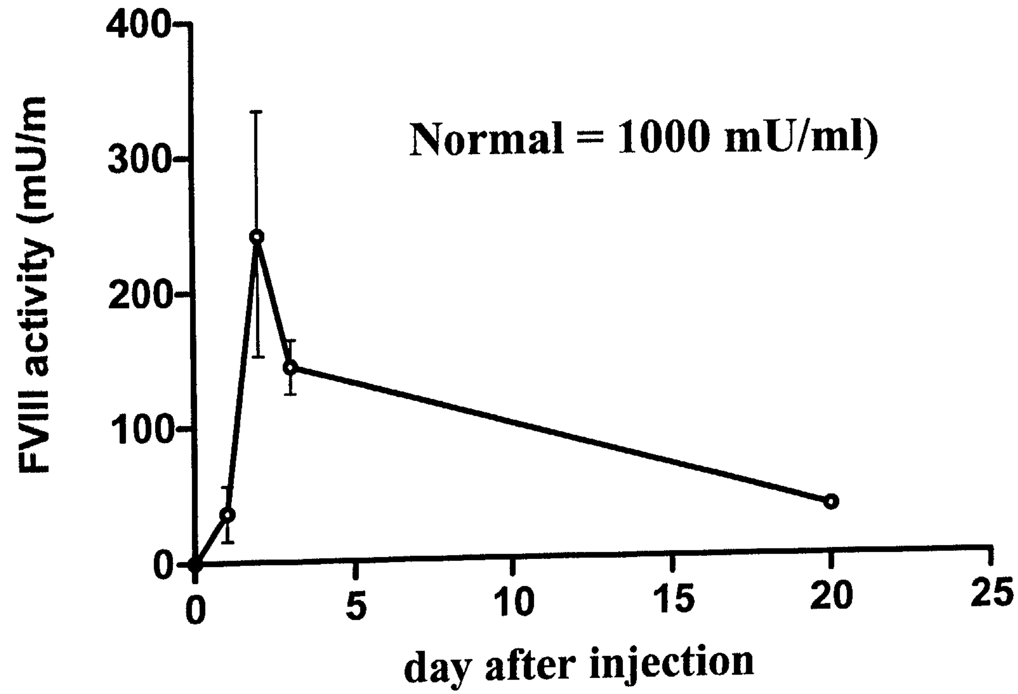


Method:
Excise TSD and part of exon 16 with
XhoI and PflMI and ligate in a PCR product that:
1) eliminates the TSD and splice acceptor site
2) inserts EcoRV adjacent to exon 16
3) restores the coding for exon 16

Repair of Factor VIII

Preliminary results from one experiment

FVIII activity in Exon 16 FVIII-KO mice after IV PTM-FVIII intraportal infusion (100ugDNA)(n=3)

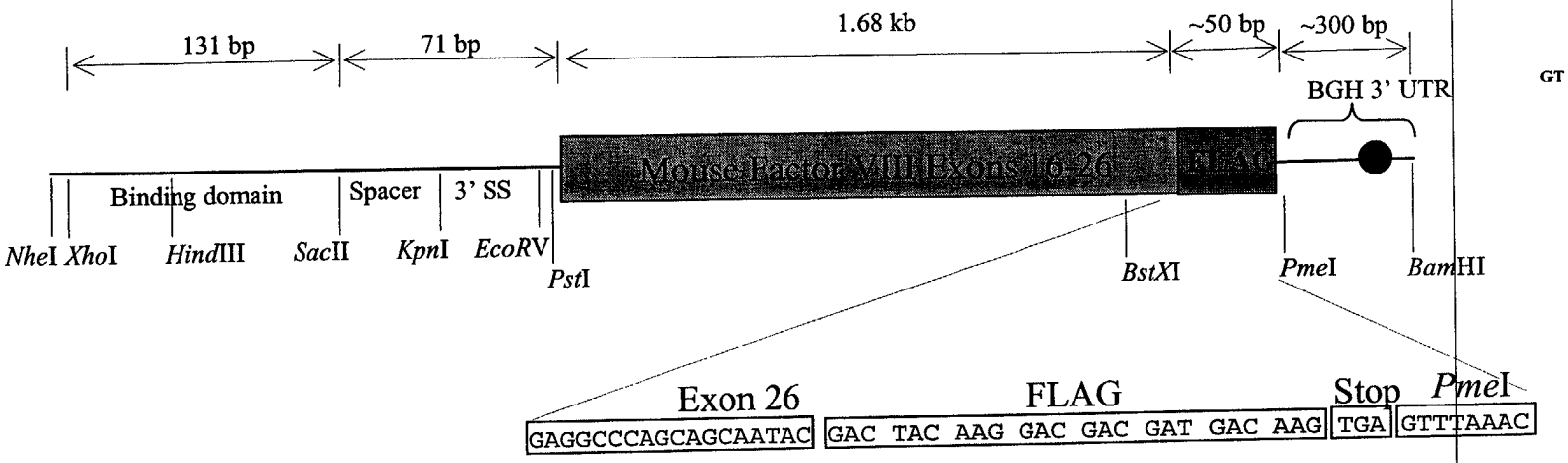


METHODS

Inject plasmid intraportally
↓
Sample blood (1, 2, 3, 20 d)
↓
Assay for factor VIII activity

Figure 46

Detailed structure of a mouse factor VIII PTM containing normal sequences for exons 16-26 and a C-terminal FLAG tag. BGH = bovine growth hormone 3' UTR; Binding domain = 125 bp.



REFERENCE FOR DESIGN OF FLAG TAG

Brann T, Kayda D, Lyons RM, Shirley P, Roy S, Kaleko M, Smith T.
Adenoviral vector-mediated expression of physiologic levels of human factor VIII in nonhuman primates.
Hum Gene Ther 1999 Dec 10;10(18):2999-3011
Genetic Therapy, Inc., a Novartis Company, Gaithersburg, MD 20878, USA.
Epitope-tagged B domain-deleted human factor VIII cDNA (flagged FVIII) was evaluated in nonhuman primates.

Figure 47A

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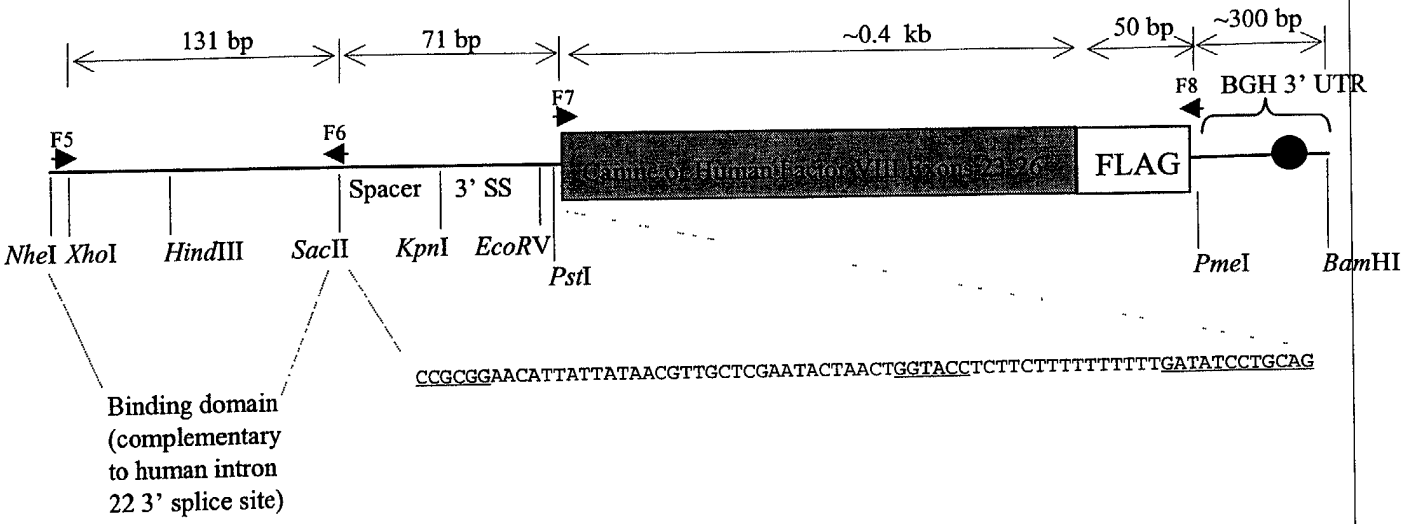

FLAG = C-terminal tag to be used to detect repaired factor VIII protein.

Figure 47B

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